Neurotrophic factors GDNF and NRTN: from basic properties to clinical trials

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It's a dangerous business, Frodo, going out your door.
You step onto the road, and if you don't keep your feet,
there's no knowing where you might be swept off to.

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LIST OF ORIGINAL PUBLICATIONS

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I **Piccinini E**, Kalkkinen N, Saarma M, Runeberg-Roos P. (2013) *Glial cell line-derived neurotrophic factor: characterization of mammalian posttranslational modifications*. Ann Med. **45**:66-73.

II Runeberg-Roos P, **Piccinini E***, Penttinen A-M*, Mätlik K, Heikkinen H, Kuure S, Bespalov MM, Peränen J, Garea-Rodríguez E, Fuchs E, Airavaara M, Kalkkinen N, Penn R, Saarma M. *Developing therapeutically more efficient Neurturin variants for treatment of Parkinson's disease*. Manuscript.

III **Piccinini E**, Saarma M, Runeberg-Roos P. *Role of heparin binding in the life cycle of NRTN*. Manuscript.

*equal contribution

The candidate's contributions include:

I. PCR mutagenesis of the GDNF plasmids to introduce the mutation N49A, designing and performing RET phosphorylation experiments (including data not shown), designing and performing glycosylation- and stability-related experiments (including data not shown), writing/editing parts of the text, and figure preparation

II. purification of the NRTN variants, designing and performing RET phosphorylation assays (with purified and unpurified proteins), performing immunocytochemistry of NRTN variants on CHO and pgsA 745 cells, immunohistochemical staining of NRTN diffusion in rat brains, measuring NRTN diffusion volume in rats, writing/editing parts of the text, and figure preparation

III. designing and performing GFRα receptors heparin affinity chromatography, designing and performing NRTN accumulation experiments, designing and analyzing experiments relative to NRTN internalization and activity, text writing/editing and figure preparation

ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder affecting seven to ten million people worldwide. The average age of diagnosis is 60, but some forms can affect even young adults. In the US alone the direct and indirect expenses for PD exceed $25 billion each year. PD is best characterized by the death of dopaminergic neurons of the *substantia nigra pars compacta*, which causes symptoms ranging from rigidity to postural instability. As the disease progresses, other areas of the brain become affected, generating psychiatric and cognitive dysfunctions.

Current therapies effectively reduce motor symptoms of PD, but do not stop its progression. Neurotrophic factors regulate neuronal growth, differentiation, and survival, and several of them have been shown to protect and regenerate dopaminergic neurons in animal models of PD. The glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) have reached clinical trials, but they did not replicate the promising results of the preclinical studies.

Several reasons, including stability of recombinant proteins and their diffusion in the brain tissue, could explain the results of the clinical trials. Stability might have been a problem especially with GDNF, which has been delivered mostly as bacterially-produced recombinant protein. In this work we analysed GDNF produced in mammalian cells and compared it to bacterially-produced GDNF. *E. coli* produced-GDNF is less stable than mammalian GDNF. This difference is probably due to the purification/renaturation method used with the *E. coli*-produced factor. Processing and stability of GDNF are affected also by cell line and medium used for its production. In mammalian cells glycosylation of GDNF is fundamental for its processing into the mature molecule.

The diffusion problem affects both GDNF and NRTN, which do not diffuse far enough from the infusion site because of their heparin-binding properties. Heparin and the closely related heparan sulphates are abundant in the extracellular matrix and on the cell surface, and hinder the diffusion of GDNF and NRTN. The diffusion issue might not be a significant problem in the animal experiments, but might limit the results achieved with humans, who have significantly bigger brain size compared to rats and monkeys. In this work we have developed NRTN mutant variants with lower affinity for heparin and characterized their activity *in vitro* and in a unilateral 6-OHDA rat model of PD. All NRTN variants were biologically active. Especially the variant N4 showed better diffusion and rescued a higher number of dopaminergic fibres than *E. coli*-produced GDNF. Toxin-treated rats administered with N4 also showed functional recovery in behavioural assays. However, as a caveat the mutations introduced could have drawbacks influencing NRTN recycling/degradation and signalling. In this respect lack of heparin-binding could affect NRTN accumulation on the cell surface and inside the cells, therefore causing a slower initiation of the signal.

Taken together our results help understanding basic features of GDNF and NRTN, such as the roles of glycosylation and of heparin binding. They also point out several important features that have to be taken into account when producing and/or modifying growth factors for clinical use, and underlines that mammalian molecules with reduced heparin binding could be beneficial for treating PD patients.

LIST OF ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone
6-OHDA	6-hydroxydopamine
AAV	Adeno-associated virus
AGRP	Agouti-related peptide
AKT	Protein kinase B
ARTN	Artemin
BBB	Blood-brain barrier
BMP	Bone morphogenic protein
cAMP	Cyclic adenosine monophosphate
CED	Convection-enhanced delivery
CLD	Cadherin-like domain
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CS	Chondroitin/dermatan sulphates
CSPG	Chondroitin sulphate proteoglycan
DBS	Deep brain stimulation
DDC	DOPA decarboxylase
DMEM	Dulbecco’s modified Eagle's medium
DRG	Dorsal root ganglion
ECM	Extracellular matrix
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
GAG	Glycosaminoglycans
Gal	Galactose
GalNAc	N-Acetylgalactosamine
Gas1	Growth arrest specific protein 1
GDNF	Glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFP	Green fluorescent protein
GFRα	GDNF family receptor alpha
GlcA	Glucuronic acid
GlcNAc	N-Acetylglucosamine
GPi	Internal pallidum
GPI	Glycophosphatidylinositol
Grb	Growth factor receptor-bound protein
HA	Hyaluronic acid
HB-GAM	Heparin-binding growth associated molecule (pleiotrophin)
HEK	Human embryonic kidney
HS	Heparan sulphate

List of abbreviations

HSPG	Heparan sulphate proteoglycan
IdoA	Iduronic acid
KS	Keratan sulphates
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO	Monoamine oxydase
MEN	Multiple endocrine neuroplasia
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
MTC	Medullary thyroid carcinoma
N-CAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NRTN	Neurturin
PD	Parkinson's disease
PI-3-K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PNN	Perineuronal net
PSPN	Persephin
r-metHuGDNF	Recombinant methionyl human GDNF
RET	Rearranged during transfection
SFK	Src family kinase
Shh	Sonic hedgehog
SHP2	Tyrosine-protein phosphatase non-receptor type 11
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TH	Tyrosine hydroxylase
UPDRS	Unified Parkinson's disease rating scale
vg	vector genomes
Xyl	Xylose

LITERATURE REVIEW

1. Parkinson's disease and its treatment

Parkinson's disease (PD) is a slowly developing neurodegenerative disorder that is best characterized by the degeneration and death of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc). The clinical signs of PD, however, appear only after 50-70% of nigral dopaminergic neurons are lost. The largest loss of neurons seems to occur within the early phases of the disease, with only little degeneration at later time points. During the early phase also 35-75% of striatal fibres are lost (Kordower *et al*., 2013). The degeneration of these neurons causes impairment of motor function, with symptoms such as rigidity, resting tremor, slow movements (bradykinesia), postural instability, and difficulties in walking. In addition to these primary motor symptoms, patients develop also secondary motor symptoms which include freezing of gait, drooling, reduced facial expression, micrographia, and speech difficulties. As the disease progresses, additional brain areas are involved, resulting in a disruption of cognitive functions, often with dementia occurring in later stages. In addition, PD patients commonly present also signs of depression, lack of motivation, sleep disorders, and several other non-motor symptoms, some of which often manifest before motor symptoms (Thomas and Beal, 2007). PD is more common in elderly people: according to the Parkinson's Disease foundation the average age of diagnosis is 60 and only 4% of patients show symptoms before the age of 50, but some genetic forms have an earlier onset and can affect even younger adults (Table 1). It is estimated that seven to ten million people worldwide are affected by PD and that 60000 new cases are diagnosed every year in the US only. The direct and indirect costs of PD sum up to $25 billion dollars/year in the US only, and a patient can spend $2500/year for medicines and $100000 for surgery treatment.

One of the hypotheses on the pathogenesis of PD states that the disease develops first in the medulla oblongata/pontine tegmentum and olfactory bulb/anterior olfactory nucleus. Only later the midbrain (and especially the dopaminergic neurons in the SNpc) is involved in the progression of the disease and the motor symptoms, which are usually the first to be noticed, appear. In the last stage, also the neocortex degenerates, causing cognitive decline (Braak *et al*., 2004).

In most cases the degenerating neurons contain aggregates of proteins which include misfolded α-synuclein and are known as Lewy bodies, but at the moment it is unclear whether these aggregates are responsible for the death of the cell or have a neuroprotective function (Engelender, 2008). It has also been suggested that misfolded α-synuclein could act in a prion-like fashion and spread transneuronally (Desplats *et al.*, 2009). However, it must also be taken into account that α-synuclein might not be the cause of the disease, but just a consequence. Studies on the progression of PD have pointed out that Lewy bodies are more common in unmyelinated cells which have a long and thin axon compared to their soma (Braak *et al*., 2004). It has also been suggested a correlation between the energy expense of the cell and the presence of the aggregates, with myelinated cells using less energy to transmit impulses along their axon, and being less susceptible to the presence of Lewy bodies. Dopaminergic neurons, instead, have massive unmyelinated axon arbours, and therefore require more energy than myelinated neurons. The main implication of this is that even small perturbations of energy production can cause cell

death in this neuronal type (Pissadaki and Bolam, 2013). Moreover, myelinated cells interact with oligodendrocytes, which could protect them from degeneration (Braak *et al*., 2004).

Although in the majority (90-95%) of cases PD is a sporadic disease with unclear causes, most of the genes that are known to be mutated in the early-onset forms of familial PD are related to mitochondrial and proteasomal functions, and to protein aggregation (Table 1). Therefore mal-functioning of mitochondria, proteasomes and/or dysregulation of protein aggregation have been hypothesized also for the late-onset sporadic cases (Thomas and Beal, 2007).

Locus	Gene	Inheritance	(Probable) function
PARK1 4q22.1	α-synuclein	AD	Presynaptic protein, Lewy bodies
PARK2 6q26	Parkin	AR	Ubiquitin E3 ligase
PARK3 2p13	Unknown	AD	Unknown
PARK4 4q22.1	α-synuclein (gene triplication)	AD	Presynaptic protein, Lewy bodies
PARK5 4p13	UCH-L1	AD	Ubiquitin C-terminal hydrolase
PARK6 1p36.12	PINK1	AR	Mitochondrial kinase
PARK7 1p36.23	DJ-1	AR	Chaperone, antioxidant
PARK8 12q12	LRRK2	AD	Mixed lineage kinase
PARK9 1p36.13	ATP13A2	AR	Lysosomal type 5 ATPase
PARK10 1p32	Unknown	AD	Unknown
PARK11 2q37.1	GIGYF2	AD	Role in IGF pathway
PARK12 Xq21-q25	Unknown	Unknown	Unknown
PARK13 2p13.1	HTRA2	AD	Mitochondrial serine protease
PARK14 22q13.1	PLA2G6	AR	Phospholipase A2
PARK15 22q12.3	FBXO7	AR	Component of modular E3 ubiquitin protein ligase
PARK16 1q32	Unknown	Unknown	Unknown
PARK17 16q11.2	VPS35	AD	Trans-Golgi trafficking and recycling of membrane-associated proteins
PARK18 3q27.1	EIF4G1	AD	Translation initiation factor
PARK19 1p31.3	DNAJC6	AR	Clathrin-mediated endocytosis
PARK20 21q22.11	SYNJ1	AR	Clathrin-coated pit dynamics

Table 1. List of familial forms of Parkinson's disease (PD). Loci, genes, inheritance and probable functions are reported. Search for inherited forms of PD was performed using the Online Mendelian inheritance in man (OMIM) search engine (http://www.omim.org/). Abbreviations: AD: Autosomal dominant, AR: Autosomal recessive, ATP13A2: ATPase, type 13A2, DJ-1: Oncogene DJ1, DNAJC60: DNAJ/HSP40 homolog, subfamily C, member 6, EIF4G1: Eukaryotic translation initiation factor 4-γ, FBXO7: F box protein 7, GIGYF2: PERQ amino acid-rich with GYF domain-containing protein 2, HTRA2: HTRA serine peptidase 2, LRRK2: Leucine-rich repeat kinase 2, PLA2G6: Phospholipase A2, group VI, PINK1: Perk-induced putative kinase 1, SYNJ1: Synaptojanin 1, UCH-L1: Ubiquitin C-terminal hydrolase, VPS35: Vacuolar protein sorting 35.

Despite the cause of degeneration not being clear, much is known about the brain areas that degenerate in PD, and on the reason why this degeneration causes the symptoms. As the most prominent symptoms of PD are related to movement, and the majority of treatments focus on relieving motor problems, this paragraph will shortly deal with the circuitry involved (Figure 1). Movement impairment is due to the degeneration of the dopaminergic neurons of the SNpc. This is a region located in the basal ganglia which has a fundamental role in the circuitry regulating movement. Shortly, sensory inputs that reach the cortex enter the basal ganglia through the dorsal striatum, which projects both directly and indirectly (via the external pallidum and the subthalamic nucleus) to the internal pallidum and to the substantia nigra pars reticulata (SNpr, the efferent nuclei). These have inhibitory projections to the thalamus, which then projects back to the cortex, more precisely to the areas involved in movement. The outcome depends on the balance between the direct and indirect pathway, with the former being facilitating on the thalamus, and the latter being inhibitory (Obeso *et al*., 2008). The dopaminergic neurons located in the SNpc project to striatal neurons. The dopamine they release regulates the activity of the striatum and it is therefore extremely important for the overall basal ganglia activity. If this is disrupted, the cortex is not able to control movement execution, and this leads to the motor symptoms observed in PD. More specifically, degeneration and death of dopaminergic neurons cause a decrease of thalamic activity. Moreover, lack of dopamine causes problems with regulation of synaptic transmission, which is mediated by neurons of the indirect pathway which express dopamine receptors (Obeso *et al*., 2008).

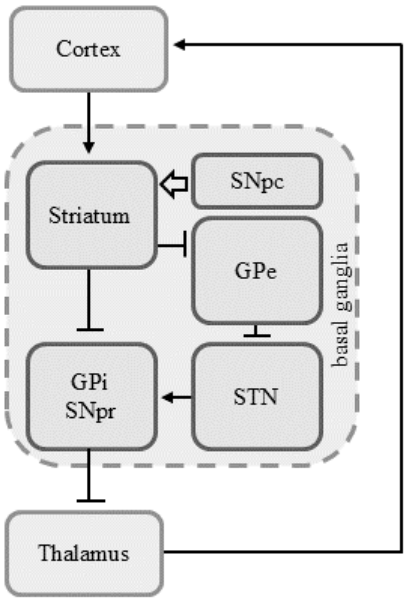

Figure 1. Schematic organization of the basal ganglia showing the most important structures and their projections. Striatum receives excitatory inputs from the cortex and has inhibitory connections to the internal pallidum (GPi)/SNpr, which inhibit the thalamus. The net result of this direct pathway is a facilitation of thalamic excitatory signalling to the cortex. Striatum also inhibits the external pallidum (GPe), which has inhibitory connections to the subthalamic nucleus (STN). This has excitatory connections to GPi/SNpr. Striatal inputs along the indirect pathway result therefore in facilitation of STN activity, leading to activation of GPi/SNpr and inhibition of thalamus. The activity of the striatum is regulated by the projection coming from SNpc. Neurons belonging to this structure release dopamine on the striatum, with effects that can be either inhibitory or facilitatory depending on the receptors present on the postsynaptic neurons.

1.1 Drug development: from animal models to clinical trials

There are several treatments available for PD patients (see section 1.2). However, these treatments act efficiently on the symptoms of PD, but do not stop or even slow down the progression of the disease. Therefore new, hopefully more effective, treatments are continuously under

development (see sections 1.3, 5 and subsections therein). When a new drug is developed, it is first tested in animal models. When the treatment has proven beneficial on animals, the clinical trial phase starts, and the drug is tested in humans. Animal models of PD have been developed for instance in *C. elegans*, drosophila, zebrafish, mouse, rat, and monkey. As the clinical trials are started only when a molecule has proven beneficial in at least two mammalian models (of which one should be a rodent and one a non-rodent species), this paragraph will deal with some of the rodent and monkey models of PD.

The most commonly used models of PD are toxin-induced. 6-hydroxydopamine (6-OHDA) was discovered more than 45 years ago, and is still widely used, especially in rats. The structure of 6-OHDA is similar to that of dopamine, but the additional hydroxyl group causes generation of reactive oxygen species, and subsequent neurotoxicity. 6-OHDA has a high affinity for both dopamine transporter and noradrenaline transporter. Therefore it is usually administered together with inhibitors of noradrenalin reuptake, in order to spare the non-dopaminergic neurons. 6-OHDA does not pass the blood-brain barrier (BBB). This is the reason why the molecule is injected in the striatum (or sometimes in SNpc) in rats, where it destroys the dopaminergic projections to striatum and the somas of dopaminergic neurons, with dose-dependent effects. 6-OHDA is effective in reducing dopaminergic fibres and amount of dopamine, and animal models show neurobehavioral defects, however, it does not replicate all of the features of PD. For instance, the animals do not show Lewy bodies or α-synuclein aggregates. Injection of 6-OHDA is performed unilaterally, because when the toxin is injected bilaterally it has severe consequences ranging from aphagia and seizures to death. Moreover, unilateral injection is useful because it allows to compare the lesioned with the unlesioned side, and allows rotational assessment of the lesion. 6-OHDA is naturally produced in the body, and it has been proposed that it could have a role in the pathogenesis of PD. However, no evidence for this has been found yet (Blesa *et al*., 2012).

Another widely used toxin model is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The importance of this molecule was discovered during 1980s, when a group of young drug addicts tried to synthesize drugs at home. They however committed mistakes during the synthesis process, which resulted in MPTP contamination and subsequent development of PD symptoms. MPTP crosses the BBB so it can be administered systemically (bilateral PD) or *via* the carotid artery (unilateral PD). MPTP is mostly used in non-human primates and mice, as rats do not respond to the toxin. MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP+) by the astrocytes and enters dopaminergic neurons through the dopamine transporter. Here, it inhibits the mitochondrial electron transport chain, leading to energy failure and oxidative stress. MPP+ is also stored in vesicles, where it is thought to cause expulsion of dopamine into the intercellular space. This dopamine is then metabolized to several compounds, including toxic ones. Some studies have shown that MPTP causes also formation of Lewy body-like structures, but they have been difficult to reproduce (Blesa *et al*., 2012).

Other studies have used herbicides like paraquat or rotenone. The structure of paraquat is similar to MPP+, and reports have shown that also their modes of action are similar. However, paraquat causes formation of Lewy body-like structures and accumulation of α-synuclein in dopaminergic neurons. Rotenone has been shown to cause damage to dopaminergic neurons, for-

mation of Lewy body-like structures, accumulation of α-synuclein in dopaminergic neurons, and gastrointestinal problems, especially after intravenous administration. Rotenone is not specific for dopaminergic neurons and affects also other brain areas, but as PD also affects several neuron types, use of rotenone is considered to recapitulate better PD pathogenesis. However, there is contrasting evidence on the depletion of dopamine in rat brains, and therefore this model is not considered to be superior to the 6-OHDA or MPTP models (Blesa *et al*., 2012).

One of the main problems of toxin- or herbicide-induced models is that they do not recapitulate exactly PD pathogenesis. PD is a slowly progressing disease, while toxin-induced lesions develop over a much shorter time. Therefore, efforts to create genetic models of PD have been made. At the moment mouse models with mutation of α-synuclein, LRRK2, PINK1, parkin, and DJ-1 are available (see Table 1 for a summary of their function). Knock-out of α-synuclein does not lead to PD-like phenotype, but the A53T mutation has been shown to cause olfactory and motor symptoms, formation of Lewy bodies, and colon dysfunction in mice. Despite LRRK2, PINK1, parkin, and DJ-1 being mutated in humans, mice models with these mutations have only mild phenotype (and sometimes no apparent phenotype at all). These models still need further development, but it has been suggested that they could be useful to study susceptibility to external factors (Blesa *et al*., 2012).

Toxin-induced animal models of PD can be used to predict whether a substance has neuroprotective or neurorestorative effects. In the first case the prospective drug is administered before lesioning the animal, in the second case it is administered after the lesion has developed. The efficacy of the molecule is evaluated through several immunohistochemical and functional tests, and unilateral lesion models are especially good for this purpose, as the unlesioned side can serve as control for the lesioned side (Blesa *et al*., 2012). Immunohistochemistry is used for instance to evaluate the amount of markers such as tyrosine hydroxylase (TH)-positive neurons (i.e. of dopaminergic neurons, which express TH, the rate limiting enzyme for the synthesis of dopamine), dopamine transporter, and dopamine and its metabolites, and to calculate the dopaminergic fibre density. Toxin administration causes a decrease in these markers, but if the prospective drug is effective, the decrease is smaller (or ideally absent). However, presence of markers does not necessarily indicate that the dopaminergic system is working efficiently. For this reason it is important to perform also functional tests. Several substances are used to induce rotational behaviour in the animal model: the more severe the lesion, the more the animal will rotate. Decrease in the amount of rotations indicates a positive treatment effect. Stimulants such as amphetamine cause turning ipsilateral to the lesion, while small amounts of apomorphine cause rotations contralateral to the lesion (Von Voigtlander and Moore, 1973). The behavioural difference depends on the mode of action of the stimulants. Amphetamine causes the release of dopamine, and therefore favours the activity of the unlesioned hemisphere (and subsequent rotation towards the lesion). Apomorphine instead stimulates postsynaptic D2 dopamine receptors, which are upregulated in the denervated striatum, thereby causing rotations contralateral to the lesion (Deumens *et al*., 2002). The use of amphetamine or apomorphine depends on the expected size of the lesion: apomorphine is better at assessing maximal-sized lesions, while amphetamine is used to assess submaximally lesioned animals (Hudson *et al*., 1993). Another test that is commonly performed in rodents is the cylinder test, where animals are placed in a cylinder and the use of their paws is

observed. Due to the asymmetry of the unilateral lesion, rats tend to use more the forelimb which is ipsilateral to the lesion. If there is neurorestoration, however, both forelimbs are used equally (Tillerson *et al*., 2001). A test which is routinely performed in monkeys is the hand-eye coordination task, where a robot presents some reward to the monkey. These treats can be non-moving, or moving at slow or fast speed. The number of successfully obtained treats in each condition gives an estimate of the hand-eye combination (Woltuis *et al*., 1995).

If the substance tested is found to have beneficial effects, it can enter the clinical trials. According to the guidelines of the Food and Drug Administration, clinical trials are divided in several phases with different goals. However, sometimes two phases can be combined together. Phase 0 (which is usually combined with Phase 1) is conducted on not more than ten people, and aims at measuring the pharmacodynamics and pharmacokinetics of the drug, particularly the half-life and the bioavailability. The dose administered is subtherapeutic. Phase 1 is also conducted on a little number of people, usually healthy volunteers. The drug is administered at low but increasing doses, in order to test its safety. If the drug is safe, it reaches the Phase 2. In this phase, 10-300 patients are administered therapeutic doses of the medicine, which is tested for efficacy. Phase 2 can be further subdivided in Phase 2a (dosing requirements) and 2b (efficacy). Sometimes Phase 1 and Phase 2 are combined together. Efficacy is tested by administering the participants either the drug or placebo, and comparing the responses. Most of the prospective drugs fail during this phase. After Phase 2, the substance enters Phase 3 and is administered to a wider number of patients (1000-3000 people), usually in centres located around the world. During Phase 3 the efficacy and safety of the drug are tested again. Only after successfully completing Phase 3 a drug can be marketed. Long-term effects of drugs are monitored at this stage (Phase 4).

1.2 Available PD treatments

The most effective treatments for PD available at the moment have an effect on motor symptoms of PD, but do not stop the progression of the disease. These treatments usually try to restore dopamine signalling, and consequently the activity of the basal ganglia.

As dopamine does not cross the BBB, the most commonly used medicine is L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of dopamine, which is readily absorbed in the intestine and is carried to the brain by the bloodstream. In contrast to dopamine, L-DOPA is able to cross the BBB through a L-type amino acid transporter (Gomes and Soares-da-Silva, 1999) and is taken up and metabolized by the remaining dopaminergic neurons. L-DOPA lowers the motor symptoms to the point that they might not be noticed, but can have different side effects, ranging from nausea to hallucinations. In addition, it is administered orally and only a small part of it (1-3%) reaches the central nervous system (CNS). For these reasons, it is never administered alone, but together with peripheral DOPA decarboxylase (DDC) inhibitors that make it possible for L-DOPA to remain intact in the periphery for a longer time (Kalinderi *et al*., 2011). Inhibition of peripheral decarboxylases also prevents the conversion of L-DOPA into dopamine, which has several effects outside the CNS: for instance it increases renal excretion of sodium and decreases production of aldosterone (therefore affecting blood pressure), and reduces gastrointestinal motility and acid secretion (see for instance Goldstein *et al*., 1995). Other medicines, such as dopamine agonists or inhibitors of the enzymes that degrade central dopamine (monoamine oxidase,

MAO, and catechol-O-methyltransferase, COMT), are used to reduce the amount of L-DOPA administered. Domperidone (an anti-dopaminergic drug which does not cross the BBB) is useful against nausea (Kalinderi *et al*., 2011). Another reason why L-DOPA is never administered alone is that, after 4-6 years of chronic use, patients develop motor complications which can lead to severe disabilities. The use of dopamine agonists and inhibitors of DDC, MAO and COMT allows the use of lower dosages of L-DOPA, which is thought to delay the onset of motor complications. In Finland, L-DOPA is commonly administered as late as possible, and the therapy starts with MAO-B inhibitors (such as rasagiline) or dopamine agonists, especially in patients younger than 75 years old. L-DOPA is administered from the beginning only in older patients and people showing cognitive symptoms. Eventually, however, every patient has to be treated with L-DOPA (see Finnish best practice guidelines at http://www.kaypahoito.fi).

Another treatment option that has proven beneficial is deep brain stimulation (DBS). This technique requires the patient to undergo surgery, and is used when the usual medical treatments have severe side effects (Kleiner-Fisman *et al*., 2006). A pulse generator that interferes with neuronal activity is implanted at the target site (either STN or internal pallidum, depending on the patient). The exact mechanism through which DBS relieves the symptoms is not perfectly understood: stimulation in mice has been shown to cause astrocytes to release adenosine triphosphate (ATP). This binds inhibitory A1 receptors and has consequences similar to those of ablation, with the difference that the effects of DBS are reversible (Bekar *et al*., 2008). DBS has several side effects: haemorrhage and infection are related to the surgery itself, and a study on PD patients showed increased problems with learning, attention, and word generation (Kleiner-Fisman *et al*., 2006). Moreover, in some cases psychiatric consequences have been observed. These can also be due to misplacement of the electrodes, but repositioning, recalibrating or withdrawing the pulse generator solves such potential problems. DBS is in use for other conditions beside PD. These include chronic pain, dystonia, essential tremor, and depression, and in these cases the pulse generator is implanted in areas relevant to these conditions (Kringelbach *et al*., 2007).

There are several other pharmacological therapies available at the moment. These include duodenal administration of L-DOPA, anticholinergic biperiden, and amantadine (see Finnish best practice guidelines at http://www.kaypahoito.fi). Biperiden improves muscle rigidity and salivation, and has a positive effect on abnormal gait and tremor, and amantadine increases dopamine release, blocks its reuptake, and reduces L-DOPA side effects (Kalinderi *et al*., 2011).

In addition to pharmacological treatments, also rehabilitation and support therapies are in use. These include for instance physical therapy, which is effective especially in milder forms of the disease. Exercise is useful to improve motor performances, and for instance dancing has been shown to improve motor control and balance. Music therapy is also used in some cases. Speech therapy is important, as it improves speech quality and voice volume (which tends to get lower in PD patients). Speech therapy might have positive effects also in relation to facial expressivity and swallowing (see Finnish best practice guidelines at http://www.kaypahoito.fi).

1.3 Towards neurotrophic and neurorestorative therapies

New studies on PD are conducted continuously in order to improve the already existing treatments and to find new ones. As an example, a search on the Thomson Reuters Cortellis™

database retrieves 36 clinical trials started during 2013, and 39 new drugs in the discovery phase added during the same period. Several types of drugs have been tested during the years, and some of these studies have focused on an extremely important concept: L-DOPA and/or DBS are indeed effective in reducing the symptoms, but dopaminergic neurons continue to die as the disease progresses. Therefore efforts to find cures that would stop the degeneration of dopaminergic neurons are continuously being made.

Already during 1980's clinicians started to consider the possibility of transplanting embryonic stem cells into the brain, in order to substitute the dying neurons. During the years there has been debate on the real usefulness of transplants of embryonic neurons and neural progenitor cells, with open-label trials showing benefits (sometimes to the point of L-DOPA becoming unnecessary) but double-blind trials not confirming those results. In addition it has been pointed out that transplanted cells do not stop the disease progression, and also the grafted neurons will eventually develop the pathology and die. However, further studies have shown that the degree of graft pathology is different in different patients, with one case still showing unaffected neurons after 14 years. Because of these conflicting results, research on this field slowed down a few years ago, but it is now being considered again, provided the techniques used to produce and implant neurons will improve (for a list of references, see Lindvall, 2013).

Another PD treatment option that has been considered suitable is gene therapy. Some of the genes that have been delivered encode for enzymes related to the synthesis/catabolism of dopamine, like TH, the rate-limiting enzyme in the synthesis of dopamine. Other genes increase the endogenous levels of neurotrophic factors, proteins which are produced in extremely limited amounts in the brain and are responsible for neuronal growth, survival, and maintenance. Related to this, is the possibility to deliver directly neurotrophic factors that are known to protect dopaminergic neurons and promote their survival. Several neurotrophic factors have been evaluated during the years. These include the basic fibroblast growth factor (bFGF), the epidermal growth factor (EGF), the brain-derived neurotrophic factor (BDNF), and the glial cell line-derived neurotrophic factor (GDNF), which has so far been the most widely used growth factor in animal models of PD (Lawlor and During, 2004). GDNF and the closely related molecule neurturin (NRTN) have reached the phase 2 clinical trials (see sections 5.1 and 5.2) and will be described in more detail in sections 2.1 and 2.2.

2. GDNF family ligands (GFLs): structure, signalling and function

GDNF family ligands (GFLs) are conserved neurotrophic factors belonging to the transforming growth factor β (TGFβ) superfamily, despite very low sequence homology with the other members of this group. All of them are dimers containing a cysteine (Cys) knot which stabilizes the structure of the two composing monomers and the resulting dimer. There are three intramolecular disulphide bonds and one intermolecular bond formed by the fourth Cys of each of the monomers (Butte, 2001).

There are four members in the GFL family: glial cell line-derived neurotrophic factor (GDNF, Lin *et al*., 1993), neurturin (NRTN, Kotzbauer *et al*., 1996), artemin (ARTN, Baloh *et al*., 1998, Rosenblad *et al*., 2000) and persephin (PSPN, Milbrandt *et al*., 1998), which all act as homodimers. The crystal structures of GDNF and ARTN have been resolved (Eigenbroth and Gerber, 1997, Silvian *et al*., 2006). GDNF and ARTN have two finger-like structures which are composed of two and four β-strands, respectively, and are separated by an α-helix which forms a region called heel (Figure 2).

Figure 2. Model of the three-dimensional structure of the glial cell line-derived neurotrophic factor (GDNF). The two monomers forming the molecule are coloured in light blue and pink, and the disulphide bonds are represented in yellow and purple. The fingers and the heel have been also indicated. Picture kindly provided by Vimal Parkash.

The receptor complex of GFLs is composed of a glycophosphatidylinositol (GPI)-anchored receptor called GDNF family receptor alpha (GFRα) and a transmembrane tyrosine kinase receptor named RET (REarranged during Transfection), and is depicted in Figure 3. There are four different GFRα coreceptors (GFRα1-4), and each of them is specific for a GFL, although there is for instance some cross-talk between GDNF and NRTN and their respective receptors (Airaksinen and Saarma, 2002). In addition, GFLs can signal through other specific molecules present on the cell surface. For instance, they can signal GFRα-dependently through the neural-cell adhesion molecule (N-CAM, Paratcha *et al*., 2003), or signal directly via syndecan-3 (Bespalov *et al*., 2011).

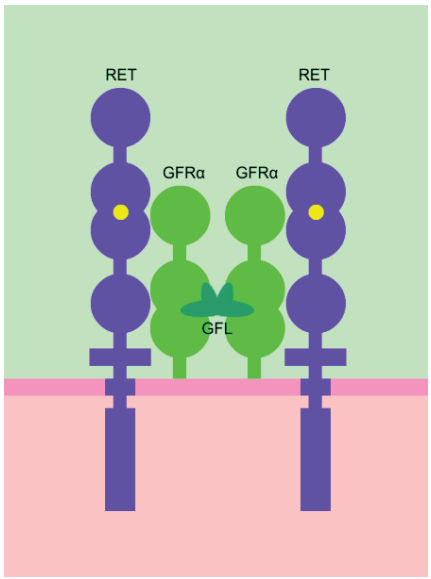

Figure 3. Model of the GFL receptor complex. The receptor complex is composed by a GFRα dimer (light green) and a RET dimer (purple). The GFL dimer (dark green) binds the co-receptor GFRα, thus initiating RET-mediated signalling. The yellow dot represent Ca^{2+} bound to RET.
Abbreviations: GFL: GDNF family ligand, GFRα: GDNF family receptor alpha, RET: Rearranged during transfection

The residues that play a role in binding of the receptor are located on the fingertips (Eketjäll *et al*., 1999, Baloh *et al*., 2000, Wang *et al*., 2006, Parkash *et al*., 2008), while the long N-terminal sequence present in GDNF does not seem to be important for binding to the receptor complex (Alfano *et al*., 2007). All GFLs are produced as pre-proproteins, from which first the pre- and then the prosequence are supposed to be cleaved (Airaksinen and Saarma, 2002).

GFLs have a role in the survival of different neural populations, including dopaminergic, enteric, sensory, motor, sympathetic, and parasympathetic neurons (Airaksinen and Saarma, 2002). In addition they are important also outside the nervous system. For instance, at least GDNF and NRTN are important in kidney development (Davies *et al*., 1999, Costantini and Shakya, 2006).

2.1 Glial cell line-derived neurotrophic factor (GDNF)

GDNF was first isolated from a rat glial cell line, on the basis of its capacity to promote survival of dissociated embryonic dopaminergic neurons and dopamine uptake in midbrain cultures. The study showed that GDNF is N-glycosylated and contains disulphide bonds. Nevertheless when the recombinant protein was produced in *E. coli*, purified, and subsequently renaturated, the resulting unglycosylated protein was active *in vitro*. GDNF increased the survival of dopaminergic neurons from dissociated rat embryonic midbrains, increased the dopamine uptake of TH-positive neurons, and increased their morphological differentiation and neurite outgrowth (Lin *et al*., 1993). In addition to the aforementioned effects on dopaminergic neurons, GDNF has several other functions, including survival, proliferation and migration of several neuron types (Sariola and Saarma, 2003). GDNF has roles also outside the nervous system: it is for instance important for the development of kidneys (Costantini and Shakya, 2006) and for regulation of spermatogenesis (Meng *et al*., 2000).

The importance of GDNF and its signalling can be seen in mice lacking the protein. Homozygote GDNF knock-outs are able to suckle and move, but die shortly after birth. They lack enteric nervous system (ENS) and kidneys, and have deficits in dorsal root ganglion (DRG), sympathetic, nodose and motor neurons. Interestingly, homozygous GDNF knock-outs do not show degeneration of dopaminergic neurons at this time point (Moore *et al*., 1996, Pichel *et al*., 1996, Sánchez *et al*., 1996). Heterozygous GDNF knock-out mice survive, but have deficits in

spermatogenesis (Meng *et al*., 2000) and learning (Gerlai *et al*., 2001) in adult age. This demonstrates that GDNF is not required for development of dopaminergic neurons, but does not explain if it is required for their postnatal maintenance. In an attempt to solve this question, grafts of dopaminergic neurons from the ventral mesencephalon of GDNF knock-outs and heterozygotes were transplanted to the denervated striata of wild type adult mice. Results showed that grafts from heterozygotes or wild type mice have the same amount of dopaminergic neurons, while grafts from homozygotes have fewer dopaminergic neurons (Granholm *et al*., 2000). Apparently contradictory results were obtained in a study on conditional GDNF knock-out mice, where GDNF expression was ablated during adulthood. In this study, mice carrying a floxed *Gdnf* allele were bred with heterozygous GDNF knock-out mice carrying the *Cre* (cause recombination) recombinase under a ubiquitous promoter. The resulting $GDNF^{F/-}$;Cre mice were administered tamoxifen at two months of age, and killed 1, 3 or 7 months later. Tamoxifen-induced recombination caused excision of 82% of the floxed *Gdnf* alleles, which resulted in levels of GDNF protein of about 40% compared to wild type mice. This decrease resulted in dramatic loss of dopaminergic neurons in SNpc and ventral tegmental area, and complete loss of noradrenergic neurons in *locus coeruleus* (Pascual *et al*., 2008). The amount of GDNF protein in this latter study is roughly comparable to the amount of GDNF in heterozygous GDNF knock-out mice. However, the results of Pascual and colleagues (2008) seem to contrast those of Granholm and colleagues (2000), where no difference between heterozygotes and wild type mice was observed. This discrepancy could be explained by hypothesizing the establishment of a compensatory neuronal pathway during embryonic development in GDNF knock-out mice.

Alternative splicing of *Gdnf* mRNA gives rise to two proteins, α-proGDNF and β-proGDNF, which differ in the length of their prosequences. These are expressed at different levels in various tissues (Suter-Crazzolara and Unsicker, 1994, Grimm *et al*., 1998), located also outside the CNS. A third variant lacking the entire exon 2 has been identified in human embryonic kidney 293 (HEK 293) cells, but it might be specific for that cell line (Grimm *et al*., 1998). Both the longer and the shorter GDNF isoform (α-proGDNF and β-proGDNF, respectively) are secreted, but through different mechanisms. The secretion of β-proGDNF increases in a Ca^{2+}-dependent manner when cells are stimulated with KCl. β-proGDNF localizes within vesicles of the regulated secretory pathway. α-proGDNF instead localizes mostly within the Golgi, and its secretion is neither depolarization- nor Ca^{2+}-dependent (Lonka-Nevalaita *et al*., 2010).

GDNF, differently from the other GFLs, has a long N-terminus which contains many basic residues. These amino acids form a heparin-binding consensus sequence (Cardin and Weintraub, 1989, Hileman *et al*., 1998, Alfano *et al*., 2007). In addition to heparin, GDNF binds also the closely related heparan sulphates (HS), for example the transmembrane heparan sulphated proteoglycan (HSPG) syndecan-3 (Bespalov *et al*., 2011). Some studies suggest that binding to HS is fundamental for GDNF signalling (Barnett *et al*., 2002), however, GDNF mutant variants completely lacking the heparin-binding N-terminus are also fully active (Alfano *et al*., 2007, Leung *et al*., 2012). In addition, the N-terminus of GDNF is important for binding to SorLA (Glerup *et al*., 2013), which is involved in recycling/degradation of the GDNF/GFRα1/RET complex.

Mature GDNF acts as a dimer and binds preferentially the GFRα1 receptor, signalling then inside the cell via RET (Jing *et al*., 1996) or N-CAM (Paratcha *et al*., 2003). Binding to GFRα1, occurs via 14 specific amino acids located on the finger structures (Parkash et al., 2008). GDNF can also bind syndecan-3 (Bespalov *et al*., 2011) and act independently of GFRα1.

As the pro-forms of some neurotrophic factors, like the brain-derived neurotrophic factor (BDNF) and the nerve growth factor (NGF), are known to have a biological activity which differs from that of the mature forms, it is surprising that only few studies have focused on the biological role of proGDNF. These studies proved that a peptide derived from the prosequence of GDNF has biological activity and can protect dopaminergic neurons (Bradley *et al*., 2010, Kelps *et al*., 2011), and that proGDNF can be secreted upon overexpression (Lonka-Nevalaita *et al*., 2010).

2.2 Neurturin (NRTN)

NRTN (Kotzbauer *et al*., 1996) was first discovered based on its ability to promote the survival of superior cervical ganglion neurons isolated from neonatal rats. Like GDNF, NRTN is important for survival of dopaminergic neurons (Horger *et al*., 1998), but there might be differences in their roles, and there are differences in the expression pattern of these growth factors. For instance, in adult mice *Gdnf*, but not *Nrtn*, mRNA can be detected in the substantia nigra (Golden *et al*., 1998). No extensive studies on the localization of the endogenous protein are available. Like GDNF, NRTN has also roles outside the nervous system. However, the molecules might act in a different way: for instance, NRTN can induce kidney branching in culture (Davies *et al*., 1999), but NRTN knock-out mice are viable and have morphologically and functionally normal kidneys (Heuckeroth *et al*., 1999). In addition, unlike GDNF which is a paracrine morphogen, NRTN is expressed directly by the developing kidney ducts, and is therefore an autocrine factor (Davies *et al*., 1999). Another difference is in the phenotype of NRTN knock-out mice (Heuckeroth et al., 1999): they have deficits in the enteric, parasympathetic and sensory systems, but differently from the GDNF knock-outs are viable and fertile.

NRTN protein sequence has 42% homology with GDNF (Kotzbauer *et al*., 1996). The biggest difference is at the N-terminus, which in GDNF is very long. As GDNF binds heparin mainly because of the amino acids present in its long N-terminal extension (Alfano *et al*., 2007), the high affinity of NRTN to heparin must be due to residues located elsewhere.

As all other GFLs, NRTN is encoded as preproprotein. However, proNRTN is poorly secreted, and is apparently not binding neither GFRα1 nor GFRα2 complexed with RET. A NRTN construct where the signal peptide is replaced with that of the immunoglobulin G heavy chain and which lacks the prosequence has been shown to be secreted much more efficiently than the wild type protein (Fjord-Larsen *et al*., 2005).

Not much is known about the role of proNRTN. However, a mutation around the cleavage site of NRTN has been linked to Hirchsprung's disease, a disease characterized by lack of neurons in the distal segments of the ENS. This mutation causes the substitution of the first amino acid of mature NRTN, changing it from alanine (Ala) to serine (Ser), and could alter the cleavage of NRTN. The substitution itself does not cause the disease, but it has been found in three affected subjects from the same family that also had a mutation in the RET gene (Doray *et al*., 1998).

Mature NRTN acts as a dimer and binds preferentially to the GPI-anchored receptor GFRα2, signalling through RET (Baloh *et al*., 1997, Klein *et al*., 1997). The presence of GFRα2 also increases the interaction of NRTN with N-CAM (Paratcha *et al*., 2003). Like GDNF, NRTN can signal also directly via syndecan-3 (Bespalov *et al*., 2011), and probably NRTN signal can be transduced by integrin β-1 too (Schmutlzer *et al*., 2011).

2.3 Artemin (ARTN)

ARTN was discovered based on sequence homology with NRTN. This factor promotes survival of a larger number of DRG and trigeminal ganglion neurons compared to GDNF or NRTN (Baloh *et al*., 1998, Rosenblad *et al*., 2000). ARTN is the only known ligand for GFRα3, which is expressed by nociceptors. For this reason, the growth factor has been studied also in relation to neuropathic pain (for a review, see Sah *et al*., 2005). ARTN also promotes the survival of E14 rat embryonic dopaminergic neurons of the ventral mesencephalon cultured for three days *in vitro*. Analysis of ARTN expression in rat brains shows that *Artn* mRNA is not expressed in the ventral mesencephalon at this stage. However, there are species-specific (and possibly developmental) differences, and in humans *Artn* mRNA is expressed at low levels in the brain, especially in the basal ganglia and thalamus. Outside the nervous system *Artn* mRNA is present, for instance, in the pituitary gland, placenta and trachea (in adults), and in fetal kidneys and lungs (Baloh *et al*., 1998).

ARTN is also initially produced as preproprotein. The propeptide is predicted to be 39 amino acids long, and it contains two additional predicted N-terminal furin cleavage sites. A putative N-glycosylation site is located at the C-terminus (Rosenblad *et al*., 2000).

ARTN acts as a dimer and signals by activating RET through GFRα3 (Baloh *et al*., 1998). The crystal structure of ARTN complexed with GFRα3 has been solved (Silvian *et al*., 2006). ARTN can also signal independently of RET, via GFRα3/N-CAM (Schmutzler *et al*., 2011). In addition, ARTN is the GFL which binds heparin with the highest affinity (Alfano *et al*., 2007), and can bind directly syndecan-3 (Bespalov *et al*., 2011).

2.4 Persephin (PSPN)

PSPN (Milbrandt *et al*., 1998) was identified using polymerase chain reaction (PCR). It was shown to promote survival of dopaminergic neurons, and their restoration after 6-OHDA lesion. In addition it is important for survival of motor neurons *in vitro* and *in vivo*. Outside the nervous system it was shown to promote ureteric branching in kidney explants (Milbrandt *et al*., 1998).

Mouse neural progenitor cells (c17.2, originating from cerebellum) overexpressing PSPN have been transplanted in striata of mice models of PD to study the function and therapeutic effects of this factor *in vivo*. These cells were found to diffuse within the striatum and to give rise to neurons, astrocytes and oligodendrocytes. Neuroprotective effects were shown in 6-OHDA-treated animals. Moreover, mice transplanted with the PSPN-overexpressing cells, but which did not receive the toxin, had better motor performance in dopamine-dependent tasks (Åkerud *et al*., 2002).

Mature PSPN binds GFRα4 causing RET activation (Enokido *et al*., 1998, Lindahl *et al*., 2000, Lindahl *et al*., 2001). Differently from the other ligands, PSPN does not bind heparin, and therefore does not signal through syndecan-3 (Bespalov *et al*., 2011). However, PSPN has been shown to bind GFRα1 (Sidorova *et al*., 2010) and signal through N-CAM (Paratcha *et al*., 2003).

Figure 4. Alignment of human GFLs. Red: presequence, light blue: prosequence, green: finger structures, **C**: cysteines involved in the knot, NXX: putative N-glycosylation sites, X: heparin-binding region, according to Alfano *et al*., 2007 (GDNF) and Silvian *et al*., 2006 (ARTN), the red vertical bars indicate predicted furin cleavage sites. The sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and then color-coded to mark Cys, N-glycosylation sites (predicted with NetNGlyc, http://www.cbs.dtu.dk/services/NetNGlyc/), pre- and prosequences, and putative furin cleavage sites (predicted with ProP, http://www.cbs.dtu.dk/services/ProP/). Abbreviations: ARTN: artemin, GFLs: GDNF family ligands, GDNF: Glial cell line-derived neurotrophic factor, NRTN: neurturin, PSPN: persephin

3. GFL receptors: structure and mediation of GFLs signalling

3.1 GDNF family receptor alpha 1-4 (GFRα1-4)

The four GFRα co-receptors are the main co-receptors for GFLs. Each of them has a preferred ligand (Figure 5): GFRα1 binds preferentially GDNF (Jing *et al*., 1996), GFRα2 NRTN (Baloh *et al*., 1997, Buj-Bello *et al*., 1997, Jing *et al*., 1997, Klein *et al*., 1997, Suvanto *et al*., 1997), GFRα3 (Jing *et al*., 1997) ARTN (Baloh *et al*., 1998, Rosenblad *et al*., 2000), and GFRα4 (Thompson *et al*., 1998) PSPN (Enokido *et al*., 1998, Lindahl *et al*., 2000, Lindahl *et al*., 2001). However, cross-talk has been reported: NRTN can bind GFRα1 and signal through it, and the same is true for GDNF and GFRα2 (Baloh *et al*., 1997, Creedon *et al*., 1997, Jing *et al*., 1997, Sanicola *et al*., 1997, Trupp *et al*., 1998, Cik *et al*., 2000). Also ARTN and PSPN seem to be able to bind GFRα1, at least in the presence of RET, which is known to stabilize the receptor complex (Carmillo *et al*., 2005, Sidorova *et al*., 2010).

The GFRα co-receptors are GPI-anchored proteins which lack cytoplasmic domains that could transduce the signal inside the cells (Figure 5). The presence of of RET or of another receptor with an intracellular domain is therefore fundamental for GFLs to have an effect. However, GFLs do not bind RET directly (see for instance Jing *et al*., 1996), and binding to GFRα co-receptors is essential for RET signalling.

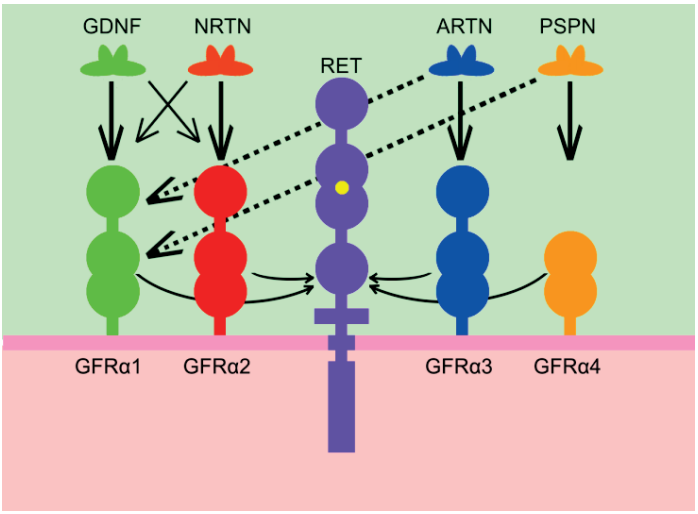

Figure 5. Binding of GFLs and GFRα co-receptors. Preferential binding is indicated with a thick, straight, arrow: GDNF binds GFRα1 (green), NRTN GFRα2 (red), ARTN GFRα3 (blue), and PSPN GFRα4 (orange). Less efficient binding, is indicated with a thin, straight, arrow: GDNF can bind also GFRα2, and NRTN can bind also GFRα1. Binding which occurs only in the presence of RET (purple) or which is still under debate is indicated with broken arrows: both ARTN and PSPN have been reported to bind GFRα1. The yellow dot represents Ca^{2+} bound to RET. The curved arrows indicate binding of the GFL/GFRα complex to RET. For clarity, only one GFRα co-receptor and one RET receptor are shown.
Abbreviations: ARTN: artemin, GDNF: glial cell line-derived neurotrophic factor, GFL: GDNF family ligand, GFRα: GDNF family receptor alpha, NRTN: neurturin, PSPN: persephin, RET: rearranged during transfection.

Mice lacking GFRα1 have almost the same phenotype as GDNF knock-out mice. They show deficits in the same subpopulations of neurons, they lack the ENS below the stomach, and the kidneys are absent. In addition, their dissected dopaminergic neurons do not respond to GDNF or NRTN in the culture medium, unless they are exposed at the same time to exogenous soluble GFRα1 (Cacalano *et al*., 1998). As mentioned earlier, GDNF can bind GFRα2, however,

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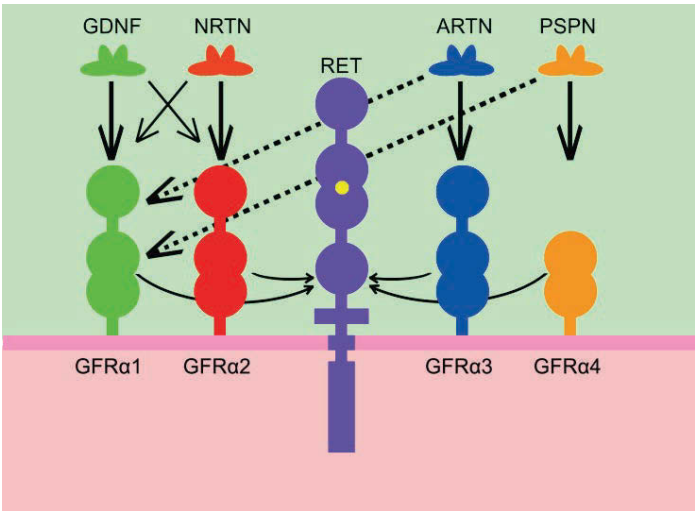

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at least in developing and adult mice (Widenfalk *et al*., 1997, Wang *et al*., 2000) and in adult rats (Marco *et al*., 2002), the dopaminergic neurons do not seem to express GFRα2. This is the reason why they cannot be rescued by GDNF or NRTN alone. Nevertheless, it has been shown that when dopaminergic neurons derived from GFRα1 knock-out mice are exposed to soluble GFRα2, they become responsive to both GDNF and NRTN (Wang *et al*., 2000). Full knock-out of GFRα2 instead leads to viable mice with a phenotype similar to homozygous NRTN knock-out mice (Rossi *et al*., 1999). Knock-out of GFRα3 suggests that the receptor might have a role in colorectal sensitivity (Tanaka *et al*., 2011), and GFRα4 knock-outs have reduced thyroid calcitonin levels, which leads to increased bone formation (Lindfors *et al*., 2006). No effect has been observed on the dopaminergic system in the two latter cases.

GFRα1-3 consist of three domains (D1-D3) with conserved Cys residues (Jing *et al*., 1996, Jing *et al*., 1997, Suvanto *et al*., 1997, Thompson *et al*., 1998). GFRα4 lacks D1, except in chicken, and is therefore shorter than the other members of the family (Lindahl et al., 2000). D1, when present, is linked to D2 *via* a hinge region, while the space between D2 and D3 is much shorter. These latter domains, but not D1, are important for ligand binding and specificity (Scott and Ibáñez, 2001). D2 and D3 have a similar structure with five α-helices and five disulphide bridges (Leppänen *et al*., 2004). The first, second and fourth helices form a triangular spiral, while the third packs antiparallelly against the second, and the fifth is a continuation of the fourth. Preliminary mutation studies (Scott and Ibáñez, 2001) predicted that the residues 211MLF and 224RRR located in D2 are important for GDNF binding. Further studies (Leppänen *et al*., 2004) have shown the importance specifically of residues 213, 224, and 225 and 229; mutation of the first three leads to lower affinity for GDNF, while the last completely abolishes the binding of the molecule and the activation of RET. The same study also found that Arg217 is probably involved in binding to RET. Studies on ARTN (Wang *et al*., 2006) complexed with GFRα3 have shown that D2 and D3 are actually part of the same globular domain which they form due to tight hydrophobic interactions. The residues involved in these hydrophobic interactions are conserved in all GFRα co-receptors, and it has been shown (Parkash *et al*., 2008) that the same globular domain composed by D2 and D3 is present also in GFRα1: D3 stabilizes D2 that is consequently able to bind GDNF.

D1 and D2 contain positively charged sequences which probably mediate heparin binding. It has been shown (Parkash *et al*., 2008) that GFRα1 binds heparin with high affinity. This affinity decreases without disappearing in mutants lacking D1. Besides this, and the predicted presence of four Cys bridges, the structure of D1 is currently unsolved and its role is still not clear. In GFRα1 at least, D1 appears to be not fundamental for binding GDNF, but its presence stabilizes the interaction of the ligand with the receptor (Virtanen *et al*., 2005). D1 does not seem to be involved in RET binding either (Virtanen *et al*., 2005); however it appears like the interaction of this domain with the ligand occurs only after the GFL-GFRα-RET complex has formed (Amoresano *et al*., 2005).

The GPI-anchor can be cleaved by the phosphoinositide-specific phospholipase C (PLC), generating soluble forms of the receptor (Yu *et al*., 1998). This indicates the possibility that GFRα co-receptors signal not only in *cis* but also in *trans*. It has been shown that GFRα1 is cleaved in several types of cells, including neurons and Schwann cells. In the presence of GDNF,

soluble GFRα1 potentiates the signalling including recruitment of RET to lipid rafts (Paratcha *et al*., 2001).

GFRα co-receptors are expressed in many different brain regions and neuron types; here I am focusing on the areas of interest for PD. It has been shown that both *Gfrα1* and *Ret* mRNA are expressed in the SNpc and striatum of both healthy adults and PD patients (Walker *et al*., 1998, Bäckman *et al*., 2006), supporting the idea of using GDNF and NRTN for the treatment of PD. However, expression of the receptors may vary depending on the species or developmental stage. For instance, in embryonic and adult mice *Gfrα1* mRNA is expressed in dopaminergic neurons in SNpc, while *Gfrα2* mRNA was found in the substantia nigra, but not specifically in dopaminergic cells (Widenfalk *et al*., 1997). Further analysis of *Gfrα2* mRNA expression shows that the co-receptor is located in cells close to the deeper migrating neurons in embryos (Wang *et al*., 2000). *Gfrα1* mRNA has been found in the striata of developing mice (Nosrat *et al*., 1997), but *Gfrα2* mRNA has not been detected in the same area (Widenfalk *et al*., 1997). Interestingly, the expression of *GFRα1* and *GFRα2* mRNAs in rat SNpc is differently regulated after a single striatal injection of 20 µg (4µl) 6-OHDA. *GFRα1* levels transiently increase one day after the lesion and then decrease three days later, while *GFRα2* expression decreases only six days after the lesion (Marco *et al*., 2002). However, data from PD patients indicate that there are no changes in the expression levels of *GFRα1* in the putamen (Bäckman *et al*., 2006).

Some GFRα receptors have also splice variants. GFRα1, for instance, has two splice variants: the longest (GFRα1a) includes a stretch of five residues (140DVFQQ) in the hinge region between D1 and D2, while the shortest (GFRα1b) does not. They are differently expressed, for instance GFRα1a is predominant in neuronal tissues and in differentiated rat adrenal pheochromocytoma (PC-12) cells, and have a different affinity for GDNF, as GFRα1b seems to bind it more efficiently. These five residues are not directly involved in ligand binding, therefore the higher affinity of GDNF for GFRα1b could actually reflect an increased stability of the complex due to a slightly different conformation of D1. The two isoforms are differently regulated during development: for instance in kidney *GFRα1b* mRNA is predominant before birth, but then *GFRα1a* takes over (Charlet-Berguerand *et al*., 2004). Also GFRα2 presents multiple isoforms: GFRα2a is the full-length isoform, GFRα2b lacks D1, and GFRα2c has the same sequence as GFRα2b, but lacks the first 28 residues after the signal sequence (Wong and Too, 1998). It has also been suggested that GFRα4 generates a GPI-anchored, a transmembrane, and a soluble isoform (Lindahl *et al*., 2000, Lindahl *et al*., 2001).

3.2 Rearranged during transfection (RET)

A mutated form of RET was first identified in human lymphoma (Takahashi *et al*., 1985), while its role in GFL signalling was discovered only 11 years later (Durbec *et al*., 1996, Trupp *et al*., 1996). Even though GFLs do not bind RET directly, this receptor is fundamental for signal transduction inside the cell. RET is heavily N-glycosylated and composed by four cadherin-like domains (CLD1-4), one Cys-rich domain, a transmembrane region and an intracellular part with a tyrosine kinase domain. The region between CLD2 and CLD3 binds a calcium-ion (Ca^{2+}) that is thought to stabilize the complex as it enables CLD1 to take contact with the ligand once the GFL has bound its GFRα co-receptor (Anders *et al*., 2001). In addition to CLD1, also CLD2 and

CLD3 have been shown to be important for binding to the GFL-GFRα complex (Kjær and Ibáñez, 2003), and likewise CLD4 and the Cys-rich domain play a role (Amoresano *et al*., 2006). The current hypothesis is that most C-terminal parts of the extracellular domain are important for taking direct contact with the GFL-GFRα complex, while CLD1-2 form a structure that stabilizes the complex (Kjær *et al*., 2010). The glycosylation level of RET does not seem to affect its binding to GFL-GFRα (Kjær and Ibáñez, 2003).

The transmembrane domain of RET is important for association of the dimer, and thereby for intracellular signalling (Kjær *et al*., 2006). The intracellular part of RET is composed of a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal tail. The juxtamembrane domain contains tyrosine and serine residues (Tyr^{687} and Ser^{696}) which are important for cyclic adenosine monophosphate (cAMP)-mediated modulation of RET activity (Liu *et al*., 1996). Indeed, mutation of Ser^{696} leads to defects in migration of enteric neurons (Asai *et al*., 2006). The kinase domain harbours several Tyr residues, and the C-terminal tail has different lengths, depending on the RET splice variant. The most studied splice variants are RET9 and RET51, which have identical N-termini. However, their C-terminal extensions starting at residue 1064 have different lengths and contain different residues. RET9 has a shorter extension of 9 amino acids, which are different from those present in the longer extension of 51 amino acids present in RET51 (Tahira *et al*., 1990). There is also a variant of RET with 43 C-terminal residues, and many more could exist based on analysis of DNA and RNA sequences (Myers *et al*., 1995).

Upon formation of the GFL-GFRα-RET dimeric complex, the intracellular domain of dimeric RET gets transautophosphorylated and initiates the signalling cascade inside the cell. There are several Tyr residues that can become phosphorylated, each of them with a different role. At the moment the most studied are Tyr 687 (located in the juxtamembrane domain), 900, 905, 981, 1015, 1062, and 1096 (Figure 6). In addition to its role in cAMP-mediated modulation of RET activity (see above), Tyr^{687} has been shown to recruit the tyrosine-protein phosphatase non-receptor type 11 (SHP2) and to be involved in mediating GDNF activity; however, for SHP2 to have effect, also the complex docked on Tyr^{1062} is essential (Perrinjaquet *et al*., 2010). It has been shown that both residues located in the catalytic site (Tyr^{900} and Tyr^{905}) are phosphorylated upon ligand binding, however, Tyr905 is the most important for downstream signalling (Kawamoto *et al*., 2004). Tyr^{905} is also the binding site of the growth factor receptor-bound protein 7 (Grb7) and 10 (Grb10, Pandey *et al*., 1996). Tyr^{981} binds the proto-oncogene tyrosine-protein kinase Src, thereby promoting GDNF-induced survival (Encinas *et al*., 2004). Tyr^{1015} binds PLCγ, and its mutation leads to decreased RET signalling and reduced oncogenic activity of the mutated form of RET formed by chromosomal rearrangements (Borrello *et al*., 1996). Tyr^{1062} can bind both Src homology 2 domain-containing protein (Shc) and the fibroblast growth factor receptor substrate 2 (FRS2) proteins (Asai *et al*., 1996), thus activating either the RAS/ERK (rat sarcoma and extracellular signal-regulated kinase, respectively) pathway and the PI-3-K/AKT (phosphoinositide 3-kinase and protein kinase B, respectively) pathway (Kurokawa *et al*., 2001), or the recruitment of Grb2 (Scott *et al*., 2005). Tyr^{1062} can bind also Dok proteins, leading for instance to neurite outgrowth in PC-12 cells (Grimm *et al*., 2001), and Enigma, causing mitogenic signalling, but the tethering of this last factor does not depend on phosphorylation (Durick *et al*., 1996). Tyr^{1096} binds Grb2, and a mutation of this residue is involved in the activity of the oncogenic form of

RET involved in multiple endocrine neuroplasia (MEN) 2B (Liu *et al*., 1996). Note that because of the different lengths of the isoforms, this last Tyr residue is present only in RET51. This is thought to be the reason why RET51 is degraded more rapidly than RET9 (Scott *et al*., 2005): it has two Tyr that can bind (directly or indirectly) Grb2, which associates with Cbl and is promoting ubiquitynation and subsequent degradation of RET.

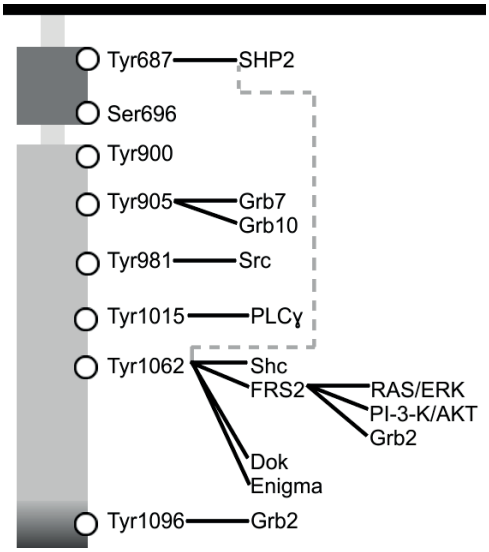

Figure 6. Schematic representation of the messenger proteins bound by the different tyrosines of the RET intracellular domain. The dark grey square represents the juxtamembrane domain, the light grey rectangle represents the tyrosine kinase domain, and its shaded part containing the residue 1096 is present only in the longer RET isoform (RET51). SHP2-mediated signalling requires interaction with complexes docked on residue 1062 (broken line).
Abbreviations: AKT: protein kinase B, ERK: extracellular signal-regulated kinase, Grb: growth factor receptor-bound protein, PI-3-K: phosphoinositide 3-kinase, PLCγ: phospholipase C gamma, RAS: rat sarcoma, RET: rearranged during transfection, Ser: serine, Shc: Src homology 2 domain-containing protein, SHP2: tyrosine-protein phosphatase non-receptor type 11, Src: proto-oncogene tyrosine-protein kinase, Tyr: tyrosine.

After activation, the receptor complex is internalized through the sortilin-related receptor SorLA. The internalization directs GDNF to the lysosomes and GFRα1 to the recycling pathway. SorLA also plays a role in the internalization of RET when GFRα1 is present, but this occurs independently of GDNF. The fate of RET has not been investigated in detail, but the internalization through SorLA does not seems to lead to increased RET degradation (Glerup *et al*., 2013). GDNF and RET are known to be retrogradely transported in neurons (Coulpier and Ibáñez, 2004, Tsui and Pierchala, 2010), but the internalization in this case is not due to SorLA, which is not present at the axon terminal (Glerup *et al*., 2013). Internalization of RET is not only important for regulating the amount of available receptor on the cell surface, but also for signalling: ERK is activated only if RET is internalized, while the AKT pathway can be activated when the receptor is on the cell surface. Activation of ERK has been linked to cell survival and proliferation, while activation of AKT is involved in cell survival and differentiation (Richardson *et al*., 2006).

RET signalling is also regulated by the growth-arrest specific protein (Gas1). This protein shows sequence similarity with the GFRα co-receptors s and inhibits GDNF signalling: RET binding to Gas1 precludes subsequent GFRα1 binding to RET. This prevents RET phosphorylation and AKT-mediated signalling (Cabrera *et al*., 2006). The same protein enhances Sonic hedgehog (Shh) signalling, and it has been shown that Gas1 mutants have deficits in the gastrointestinal system due to reduced Shh signalling and enhanced RET signalling (Biau *et al*., 2013).

RET is expressed in many different cell types. These include dopaminergic, noradrenergic and motor neurons, sympathetic, parasympathetic and sensory neurons, and the ENS. RET

mostly regulates the growth and maturation of these structures, and it is important for maintenance of dopaminergic neurons: conditional knock-out mice do not show degeneration at nine months of age (Jain *et al*., 2006), but at two years the amount of dopaminergic neurons is significantly reduced compared to wild-type mice (Kramer *et al*., 2007). Homozygous knock-down of RET is lethal in mice (Schuchardt *et al*., 1994). These mice die soon after birth due to kidney agenesis and lack of enteric neurons, a phenotype that resembles closely those of GDNF and GFRα1 knock-outs. This effect is isoform-dependent: mice lacking RET51 develop normally and do not present any obvious phenotype, but when RET9 is not present the enteric ganglia are missing from the colon and kidneys are small and underdeveloped (de Graaff *et al*., 2001).

Outside the nervous system, RET can be found for instance in ureteric buds, spermatogonia, and thyroid (Wang, 2013). This receptor is implicated in various diseases: it is constitutively active in patients affected by tumours of neural crest origin, such as neuroblastoma, pheochromocytoma, and medullary thyroid carcinoma (MTC), which affects the calcitonin-producing C cells in the thyroid. These conditions can be present alone, for instance in familial medullary thyroid carcinoma (FMTC) only MTC is present, or together with others, like in MEN syndromes. MEN2A patients show MTC, pheocromocytoma and hyperparathyroidism, while MEN2B patients display MTC, pheochromocytoma, marfanoid habitus, thickened corneal nerve, and ganglioneuromatosis of gastrointestinal tract (Santoro *et al*., 2004). In these cases the gain-of-function mutations are usually, but not always, located in the Cys-rich or in the kinase domain and lead to constitutive receptor dimerization and/or signalling (see for instance Takahashi *et al*., 1998, Iwashita *et al*., 1999). Another type of tumour where RET is constitutively active is papillary thyroid carcinoma: in this case RET gene is rearranged so that it is fused with heterologous genes (Arighi *et al*., 2005). On the contrary, in patients with Hirchsprung's disease, RET presents mutations that make it inactive and cause lack of neurons in the distal segments of the ENS (Arighi *et al*., 2005). In this case, many mutations occur on the CLDs, and they affect the folding of the molecule, its maturation and the transport to the cell membrane (for a list of references see Anders *et al*., 2001). Other relevant residues are located in the kinase domain or important for binding adaptor proteins (Iwashita *et al*., 1996, Geneste *et al*., 1999). For more comprehensive reviews about RET signalling and role in diseases, see Arighi *et al*., 2005 and Runeberg-Roos and Saarma, 2007.

3.3 Role of the neural-cell adhesion molecule (N-CAM) in GFL signalling

N-CAM is a neural cell adhesion molecule identified almost 40 years ago in neural retinas of chick embryo (Thiery *et al*., 1977). Besides cell-cell adhesion, it can mediate many other processes, including cell-matrix adhesion, neurite outgrowth, neuron-muscle interaction, synapse formation and synaptic efficacy. In addition, N-CAM expression is elevated in certain tumours, including small cell lung cancer, neuroblastoma, and rhabdomyosarcoma. Despite N-CAM being expressed both in tumours and in tissues surrounding them, research has focused on immunotherapeutic targeting of N-CAM (Jensen and Berthold, 2007). N-CAM interacts with several different ligands (Jensen and Berthold, 2007), but this paragraph will briefly deal with its role in GFLs signalling.

There are several N-CAM isoforms, but the most studied have differences only in the C-terminal part. N-CAM120 is GPI-anchored, N-CAM140 has a short intracellular domain, and N-CAM180 has a long intracellular domain (Dallerác *et al*., 2013). N-CAM120 and N-CAM140 have been shown to bind GFRα1 and GDNF. In the presence of GDNF and GFRα1, N-CAM140 is able to initiate signalling inside the cell independently of RET. Binding to N-CAM leads to reduced N-CAM-mediated adhesion, to Schwann cell migration and to axonal growth in neurons located in the cortex and hippocampus, and occurs via phosphorylation of the proto-oncogene tyrosine-protein kinase Fyn and the focal adhesion kinase (FAK), which participate in rearrangements of the cytoskeleton. N-CAM can be polysialylated (PSA), leading to an increased negative charge. PSA-N-CAM binds GDNF directly, but for signalling the presence of GFRα1 is required (Paratcha *et al*., 2003). Evidence suggests that also the other GFLs in complex with their respective GFRα co-receptors can signal through N-CAM independently of RET. For instance, it has been shown that GDNF, NRTN and ARTN induce increased release of calcitonin gene-related peptide (CGRP) in sensory neurons in the presence of capsaicin. Intriguingly, when knocking-down the expression of RET (Schmutlzer *et al*., 2011), GDNF-induced increase is essentially abolished, but the effects of NRTN and ARTN are only reduced. Further decrease is observed when also N-CAM expression is knocked-down, suggesting that NRTN and ARTN signalling is mediated not only by RET but also by N-CAM (Schmutlzer *et al*., 2011). In addition, at least upon overexpression, N-CAM can bind GFRα4, suggesting that also PSPN could signal through this alternative pathway (Paratcha *et al*., 2003).

4. Glycosaminoglycans (GAGs): roles in GFL signalling

Glycosaminoglycans (GAGs) are long linear polysaccharides composed of two repeating sugars. These sugars are usually an amino sugar, like N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine, galactose (Gal) or an uronic sugar (glucuronic acid, GlcA, or iduronic acid, IdoA). GAGs are divided into four groups, based on core disaccharide sugars: hyaluronic acid (HA), keratan sulphates (KS), chondroitin/dermatan sulphates (CS), and HS. GAGs can be transmembrane, GPI-anchored to the cellular membrane, or soluble proteins. Several types of GAGs can be linked to the same protein, for instance syndecan-3 contains both HS and CS side chains (Bovolenta and Fernaud-Espinosa, 2000). GAGs are usually synthesized in the Golgi, except for HA which is synthesized at the cellular membrane (Fraser *et al*., 1997). This is in accordance with the fact that HA is the only GAG present not only in mammalian cells, but also in bacteria, which do not have organelles, and therefore have to rely on different mechanisms for the synthesis of GAGs. All GAGs are highly polar, but HS, CS and KS are rich in sulphate groups (which are heavily negatively charged), while HA is not sulphated (Bovolenta and Fernaud-Espinosa, 2000).

GAGs can bind many different types of proteins, such as proteases and their inhibitors, chemokines, cytokines, and growth factors. As an example, heparin alone interacts with almost one fourth of the plasma proteins. GAGs are also components of the extracellular matrix (ECM), and can bind other macromolecules of the ECM. As the ECM provides structural and biochemical support to the cells, GAGs have for instance roles in cell adhesion, cell-to-cell communication, and differentiation. GAGs have also other physiological roles, including ligand binding, signalling regulation, and protection from proteolytic cleavage (Zhang, 2010).

It is not known whether GFLs bind HA or KS. Therefore the next paragraphs will present only chondroitin/dermatan sulphate proteoglycans (CSPGs) and HSPGs, particularly syndecan-3. CSs and HSs are produced in huge amounts by the cells. Their exact amounts and types vary based on the tissue, but each cell exposes 10^5-10^6 molecules on its surface, and their concentration in the ECM is in the range of mg/ml. CS and HS can be attached to proteins, forming CSPGs and HSPGs, but also hybrid proteoglycans. Each proteoglycan can host from one to more than hundred GAGs (Zhang, 2010).

4.1 Chondroitin/dermatan sulphate proteoglycans (CSPGs)

CSPGs are formed through a series of enzymatic steps (Zhang, 2010) which starts with the addition of a linker tetrasaccharide to a Ser residue in the sequence Ser-Gly/Ala-X-Gly (where X is not Pro). This link is composed of GlcA-Gal-Gal-Xyl, and the first sugar to be added in the endoplasmic reticulum is xylose (Xyl). Until this point, syntheses of CS and HS are identical, but the moiety of the fifth sugar determines what type of GAG will be formed. In the case of CS, N-acetylgalactosaminyltransferase I adds N-acetylgalactosamine (GalNAc), and then chondroitin synthase attaches GlcA and GalNAc. These form the repeating disaccharide typical of most CS except dermatan sulphate, where GlcA is replaced by IdoA (Figure 7A).

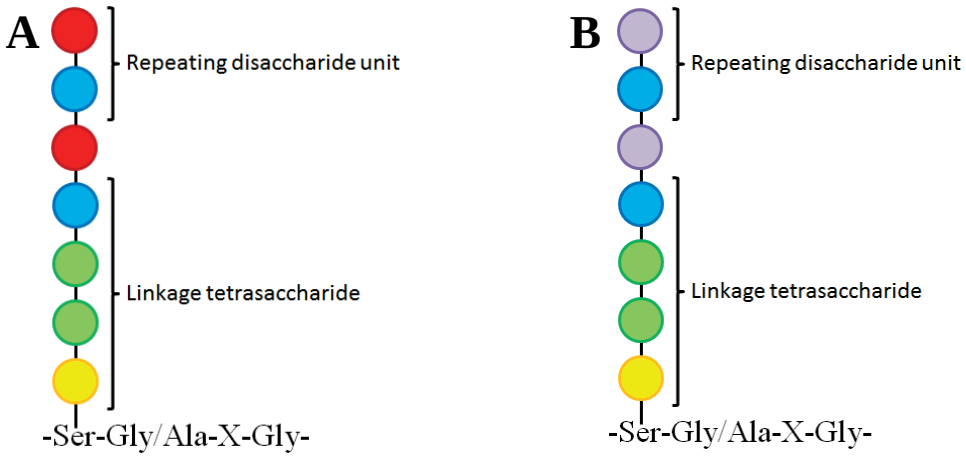

Figure 7. A. Synthesis of CS. In dermatan sulphate, the GlcA of the repeating disaccharide unit is replaced by IdoA. **B. Synthesis of HS.** Yellow: Xyl, green: Gal, light blue: GlcA, red: GalNAc, lilac: GlcNAc.

The sugar structure is then sulphated: CS have long tracts consisting of modified disaccharides. Some types of sulphation are present both in CS and HS, but others are specific for each type: for instance only CSs present GlcA-3-O-sulphation. Other modifications consist in 4-O- and 6-O-sulphation of GalNAc, and 2-O-sulphation of the uronic acid (Zhang, 2010).

CSs are typically involved in structural and regulatory activities. For instance, as components of the ECM, they are important for the integrity of tissues like cartilage (Dijkgraaf *et al*., 1995). In the CNS, CS have a regulatory role: they are for instance the fundamental components of the perineuronal nets (PNNs) in the brain and in the visual cortex. PNNs are specialized structures of the ECM which have a role in the closure of critical periods. During the critical period, sensory experience shapes the organization of the neuronal network, which is plastic at this stage. For example, in the visual cortex of young rats the PNN is not completely formed: this allows plasticity and correct organization of the neuronal connections. However, the cortex of adult rats with a well-developed PNN is less plastic. This is partially due to CSPGs which are present in adult rats, but not in younger animals. It has been shown that digestion of CSPGs with chondroitinase ABC restores the neuronal plasticity in adults (Pizzorusso *et al*., 2002). However, some CS have apparently different functions: a class of CS (CS-E) is instead promoting axonal growth in a cell-specific fashion (Mikami and Kitagawa, 2013).

GDNF binds CS with high affinity (10-fold higher than affinity to HS) and with almost no dissociation. It has therefore been proposed that CS could keep holding neurotrophic factors while presenting it to the receptor, or that growth factors could be enzymatically released from such molecules (Nandini *et al*., 2004). However, CS does not seem to be important for GDNF signalling, as this is not decreased in cells lacking CSPGs, and exogenously added CS had only a slight effect on GDNF activity (Barnett *et al*., 2002).

4.2 Heparan sulphate proteoglycans (HSPGs)

Synthesis of HSPGs starts in the same way as that of CSPGs. The difference arises when the fifth sugar is added to the linker sequence: in the case of HSs this is GlcNAc, and the repeat-

ing disaccharide unit is composed by GlcA and GlcNAc (Figure 7B), added respectively by exostosin I and II (Zhang, 2010).

Differently from CS, HS present long streaks of non-sulphated disaccharides separating clusters of modified sugars. Like CS, HS can be O-sulphated, but only CS can be modified with N-sulphation (Zhang, 2010). HSPGs can be roughly divided in three main groups: syndecans have transmembrane domains and interact with intracellular proteins, glyphicans are GPI-anchored, and other HSPGs (for instance perlecan, agrin, and collagen XVIII) are secreted, ECM-located proteins (Lindahl and Kjellén, 2013).

Because of the presence of sulphate groups, HS are highly negatively charged and can bind several types of molecules, including growth factors, morphogens and inflammatory mediators (Lindahl and Kjellén, 2013). They have therefore many different functions: form protein gradients, protect molecules from degradation, have structural roles, and are implicated in signalling, either directly or because they affect the binding of ligands to their receptors. Dysregulation of this last function has been observed in several pathologies. For instance HS-dependent Wnt signalling is upregulated in gastric cancer (Takei *et al*., 2011), and HSPG-related bone morphogenetic protein (BMP) signalling is involved in fibrodysplasia ossificans progressiva (O'Connell *et al*., 2007).

Regarding binding to GFLs, it is known that GDNF, NRTN, and ARTN, but not PSPN, have high affinity for heparin and HS (Alfano *et al*., 2007, Bespalov *et al*., 2011), and that the same is true at least for GFRα1 (Parkash *et al*., 2008). It has therefore been proposed that HSPGs are important for GFL signalling, and this has been shown both for HS (Barnett *et al*., 2002, Piltonen *et al*., 2009) and heparin (Tanaka *et al*., 2002). GDNF binding to HS depends on the 2-O- and the 6-O-sulphation of HSPGs (Rider, 2003, Bespalov *et al*., 2011). This is also reflected by the fact that the phenotype of mice lacking 2-O-sulphotransferases resembles closely that of GDNF, GFRα1 or RET knock-outs: the animals die perinatally because they lack kidneys. This is caused by failure of ureteric bud branching and condensation of the mesenchyme. These mice also show ocular and skeletal defects (Bullock *et al*., 1998). SULF1 and SULF2 are extracellular enzymes which remove 6-O-sulphation from HS. Mice with double knock-out of these enzymes have an increased GDNF binding to HS and reduced signalling in the embryonic oesophagus. As SULFs are differentially expressed, they could be important to regulate GDNF binding and subsequent activity in different tissues during development (Ai *et al*., 2007). Moreover, HSPGs may not only regulate GDNF access to GFRα co-receptors, but can themselves act as signalling receptors for the molecule. This is the case for syndecan-3 which has been shown to bind immobilized GDNF and stimulate cell spreading and neurite outgrowth via the Src family kinase (SFK). ECM-bound GDNF is therefore thought to be involved in cell migration during the development of the cortex (Bespalov *et al*., 2011).

Knock-out and/or mutation of different types of HSPGs have been performed in mice models. The effects range from not noticeable phenotypes to early embryonic death, with the most severe phenotypes appearing when HS chains are lost (Lindahl and Kjellén, 2013). However, not much is known about the role of HSPGs in PD and whether their expression is somehow altered in patients. Only one study has focused on the role of agrin, a transmembrane and extracellular HSPG, in PD. This study shows that agrin binds α-synuclein, promoting conformational

changes and aggregation, and reducing its solubility. In the same study, agrin was found to colocalize with Lewy bodies in the substantia nigra of PD patients (Liu *et al*., 2005). As Lewy bodies are located intracellularly, it would be interesting to understand whether this is due to alterations in agrin trafficking or endocytosis. Another study has focused on the matrix metalloproteases, enzymes which degrade the ECM and are released by several cell types in the CNS in response to cytokines and oxygen-free radicals. Results showed increased amounts of matrix metalloprotease inhibitors in the substantia nigra and cerebrospinal fluid of PD patients (Lorenzl *et al*., 2003). The authors hypothesize that the increased levels of inhibitors would prevent further damage to the ECM, but do not comment on the possible effects on the level of ECM proteoglycans.

4.2.1 Syndecan-3

Syndecans are transmembrane HSPGs. They are divided into several families, of which syndecan-3, also known as N-syndecan, has been shown to be important for GDNF signalling. Syndecan-3 (Carey *et al*., 1992, Gould *et al*., 1992) has an extracellular domain which harbours several GAGs. Syndecans have variable N-termini which are followed by more conserved sequences including a cleavage site and the transmembrane and cytoplasmic domains (Bernfield *et al*., 1992).

Syndecans are present in all tissues, but with a very specific distribution: for instance syndecan-3 is mostly found in neurons (Carey *et al*., 1992). Homozygous syndecan-3 knock-out mice are viable, but present altered feeding behaviour (Reizes *et al*., 2001), higher long-term potentiation in the hippocampus with subsequent hippocampal memory impairment (Kaksonen *et al*., 2002), and impaired cell migration (Hienola *et al*., 2006).

Like all other HSPGs, one of the roles of syndecans is to bind ligands so that they can then be presented to their receptors. One example of such a function of syndecan-3 is related to feeding behaviour. Anti-satiety molecules, like the agouti-related peptide (AGRP), and satiety ones, as the α-melanocyte stimulating hormone (α-MSH), compete for binding to the same receptor (melanocortin-3/4 receptor). Syndecan-3 can bind AGRP, thus enhancing its interaction with this receptor and increasing hunger. However, when the extracellular domain of syndecan-3 is cleaved in response to feeding, also AGRP is removed, so that α-MSH can bind the receptor and mediate satiety (Reizes *et al*., 2001).

However, syndecans do not only participate in ligand-receptor interactions, but due to their intracellular domain can also activate different signalling pathways inside the cell, like the protein kinase C alpha (PKCα, Lebakken and Rapraeger, 1996), and SFK (Kinnunen *et al*., 1998). The way the signalling starts is not very well known, but it is thought that ligand binding triggers syndecan oligomerization/clustering. This has been proposed, for instance, in the case of the heparin-binding growth associated molecule (HB-GAM)-induced syndecan-3 activation (Rauvala *et al*., 2000). Syndecan-3 binding of immobilized (but not free) HB-GAM induces neurite outgrowth and cell spreading. This happens via SFK, which promotes activation of factors responsible for actin branching. As syndecans do not have tyrosine kinase or any other catalytic activity, it is thought that their oligomerization brings the signalling complexes bound to the cytoplasmic domain of syndecans close together. This proximity promotes transactivation of the downstream signalling effectors. (Raulo *et al*., 1994, Kinnunen *et al*., 1998).

Syndecan-3 may both help GFL-receptor interaction and act as a GFL receptor itself. Syndecans bind several different cell-adhesion molecules, such as N-CAM (Storms and Rutishauser, 1998). It is known that GDNF binds syndecan-3 (Bespalov *et al*., 2011), and N-CAM (Paratcha *et al*., 2003). Therefore, it is tempting to speculate that syndecan-3 in this case presents GDNF to N-CAM and facilitates their interaction. Moreover, it has been shown that immobilized GDNF, NRTN, and ARTN bind syndecan-3 and promote cellular migration and neurite outgrowth by activating Src kinase. This suggests a role of ECM-bound GFLs in brain development, and is supported by the observation that both GDNF and syndecan-3 knock-out mice have fewer cortical γ-aminobutyric acid–releasing neurons (Bespalov *et al*., 2011).

5. Neurotrophic factors as PD therapeutics: focus on GDNF and NRTN

Delivery of neurotrophic factors is considered to be the most promising direction at the moment. There are several reasons for this: first, many of these factors have been shown to promote neuron survival and regeneration in toxin-induced models of PD. Second, neurotrophic factors could also be useful in restoring the functions of neurons which are losing their phenotype (Peterson and Nutt, 2008). It has also been proposed that degeneration of dopaminergic neurons could be due to the loss of these neurotrophic factors or impairment of their signalling (Granholm *et al*., 2000, Kramer *et al*., 2007). All these arguments support the hypothesis that trophic factor delivery would restore signalling pathways and therefore stop or slow down neuronal degeneration. Delivery can occur in different ways: for instance as purified protein, through lentivirus or adeno-associated viruses (AAV), or via encapsulated cells secreting the factor. GDNF and NRTN have been used in clinical trials (see sections 5.1-3) and delivered as protein and *via* AAV2 vectors, respectively. The use of encapsulated, rather than naked, cells for delivering GDNF prevents migration of the cells to other structures and the risk of rejection. The techniques used to produce encapsulated cells have improved with time, but still the production of GDNF decreases when the cells are implanted *in vivo*, so this technique requires better development before it can be used in clinical trials (Lindvall and Wahlberg, 2008).

5.1 Therapeutic potential of GDNF protein delivery

Recombinant methionine human GDNF (also called liatermin or r-metHuGDNF) entered PD clinical trials more than ten years ago (Gill *et al*., 2003). This factor was produced in *E. coli* by Amgen and had the sequence of mature GDNF with an extra methionine at the N-terminus. Liatermin was used in all the listed clinical trials where GDNF was delivered as a protein (Table 2).

As GDNF does not pass the BBB, it had to be injected directly into the brain tissue of patients. Since data available from animal models (Gash *et al*., 1998) had showed that GDNF was effective when injected intraventricularly, intrastriatally, and intranigrally, the first Phase 1/2 trial (Nutt *et al*., 2003) relied on those results. GDNF was delivered into the right ventricle of PD patients. The subjects received monthly injections of either placebo or GDNF (ranging from 25 to 4000 μg) for eight months in a double-blind study. Part of them entered then an open-label trial. All patients receiving GDNF showed adverse effects like loss of appetite, nausea, depression, and Lhermitte's sign (an electrical sensation running down the spine and limbs), and none improved significantly when compared to patients treated with placebo. One of the GDNF-treated patients died three weeks after receiving the last injection, of causes unrelated to the treatment. His brain was fixed and analysed (Kordower *et al*., 1999). Results showed that TH levels in the striatum were not different from those of PD controls who had not received GDNF treatment, but they were elevated in a region going from the bed nucleus of the stria terminalis to the substantia innominata. In addition, no GDNF immunoreactivity was observed in the treated patient, while the growth factor was detectable, although in low amounts, in a monkey MPTP-model which had received GDNF injections and was used as a control in the same study (Kordower *et al*., 1999). The authors conclude that the presence of the adverse effects showed that GDNF was biologically active, but the protein had failed to reach the putamen and the SNpc, probably due to the large

brain volume (Kordower *et al*., 1999, Nutt *et al*., 2003). However, GDNF was administered to the monkeys following a different protocol, and the time between the last GDNF injection and the diffusion analysis was much shorter in the animals than in the deceased patient. Therefore the results remain unclear.

Meanwhile, GDNF injected into the striatum of a primate model of PD had been shown to be retrogradely transported along the dopaminergic axon terminals and to promote the survival of dopaminergic neurons (Grondin *et al*., 2002). Following these findings, another Phase 1 clinical trial (Gill *et al*., 2003) was started. In this case 14.4 µg/side/day of r-metHuGDNF was chronically infused for two months into the striatum of five PD patients through a catheter connected to a pump. After this time, the daily dose of GDNF was increased to 43.2 µg/side/day. One of the patients had unilateral symptoms and received the protein only on the contralateral side; the others were affected bilaterally, and received GDNF on both sides. This time, the only adverse effect was the Lhermitte's sign. In addition a region of high signal was visualized close to the catheter tip during magnetic resonance imaging (MRI). Its intensity varied between patients (and hemispheres), but was stronger at higher doses of GDNF, so the amount of neurotrophic factor injected was reduced back to 14.4 µg/side/day, as the clinicians hypothesized that the signal could be due to oedema or protein accumulation. The results were positive: PD symptoms improved after three months of infusions, to the point that there were no periods of severe immobility anymore and some patients were more responsive to L-DOPA, so they could reduce the dosage of medicine. In addition, three patients out of five recovered the sense of smell, which is often impaired in PD. The scores on the Unified Parkinson's Disease Rating Scale (UPDRS, a scale where behaviour, mood and clinical symptoms are evaluated by the clinician, and the patient self-evaluates several activities of daily life which range from swallowing to hygiene) were 48% lower than the baseline after one year of treatment. Also the sub-scores measured during daily activities had improved. At the same time, the uptake of ^{18}F-DOPA in the putamen and the SNpc was increased (Gill *et al*., 2003).

A two-year follow-up (Patel *et al.*, 2005) showed that patients could tolerate continuous GDNF infusions over a long period, and that the molecule was effective in slowing down the progression of the disease. Moreover, when the patient that had received only unilateral injection died of unrelated causes, analysis showed that GDNF had induced increase in TH nerve fibres in the striatum and, possibly, also sprouting of fibres in the SNpc (Love *et al*., 2005).

The patients treated in this study were only a few, without negative control, and in addition it is known that a certain improvement is seen also in patients treated with placebo (Goetz *et al*., 2000). Nevertheless the improvement was so noticeable, that it was decided to verify the results with a Phase 2 trial (Lang *et al*., 2006). This time, 34 patients were bilaterally implanted with the catheter and randomly divided into two groups, of which one was treated with placebo and the other with GDNF (continuous infusion for a total of 15 µg/side/day). Unfortunately, the results were not as good as in the Phase 1 trial: patients treated with GDNF did not develop serious side effects (only headaches, paresthesias and upper respiratory tract infections were reported), but their condition did not improve either. In addition, some of the patients developed GDNF-neutralizing antibodies, but these did not give rise to further consequences. The authors hypothesize that the discrepancies were probably due to a placebo effect, with patients improving

more in the Phase 1 study (Gill *et al*., 2003) because they had higher expectations. Other reasons could have been the differences in dosage, in the condition of the patients (those participating in the Phase 1 trial had milder PD and therefore their neurons could have been more responsive) and in the catheter used, as the one adopted during the Phase 2 trial was smaller.

Meanwhile, another Phase 1 trial (Slevin *et al*., 2005) with a different protocol was being carried out. In this case, the catheter was inserted only controlaterally to the most affected side. The other difference between this trial and the previous one (Gill *et al*., 2003, with its continuation into Phase 2, Lang *et al*., 2006) was the delivery method, which was implemented with the goal of enhancing GDNF diffusion. They used a multiport catheter, and the protein was infused using a convection-enhanced delivery-like fashion (CED). CED-like delivery was obtained by continuous infusion at 2 µl/hour, plus small bolus injections every six hours. Ten patients receive daily doses of GDNF which were increased at eight-week intervals (starting from 3 µg/day and up to 30 µg/day), and the results were evaluated one month after the last injection. Again, the only observed side effect was Lhermitte's sign, and the patients improved significantly on both sides, showing for instance a better posture and speech than prior to the therapy. However, while the extended part of the trial was ongoing, Amgen decided to halt all the ongoing trials, due to the failure of the Phase 2 trial and to safety issues (Lang *et al*., 2006). The safety issues included the already mentioned development of GDNF-neutralizing antibodies by some of the patients (probably due to peripheral leakage), and cellular damage occurring in certain brain areas. It was later hypothesized (Salvatore *et al*., 2006, Slevin *et al*., 2007) that the damage could depend on the sudden GDNF withdrawal: delivery of exogenous GDNF could downregulate synthesis of endogenous protein, so that, after stopping the therapy, GDNF-dependent cells would die because of lack of neurotrophic factor. One year after the termination of the study, patients that had participated in the trial by Slevin *et al* were analysed again (Slevin *et al*., 2007): results showed that all the improvements were lost. This led the authors to conclude that continuous GDNF infusion is necessary to maintain the positive effects. In addition, they discuss the results of the Phase 2 trial conducted by Lang and colleagues, pointing out that the antibodies developed by some of the patients were against exogenously added recombinant GDNF (produced in *E. coli* and then reconstituted), and therefore not necessarily effective against endogenous GDNF.

These were not the only critiques to the Phase 2 study (Lang *et al*., 2006): Hutchinson *et al* (2007) reported that the standard deviation of the measurements was much higher than expected. Therefore the study was underpowered, and the results inconclusive. Moreover, a study by Salvatore *et al* (2006) used the same delivery system and protocol as in the failed Phase 2 trial to study the diffusion of ^{125}I-GDNF in monkey brains. GDNF was detected by measuring iodine signal, by immunohistochemistry and by enzyme-linked immunosorbent assay (ELISA): the results showed that GDNF was present in the putamen and retrogradely transported to the SNpc, but its concentration and volume of diffusion varied greatly between monkeys. The highest concentration of GDNF was found within a 2-mm distance from the catheter tip, and the diffusion volume ranged between 87 and 369 mm^3, enough to cover only from 2 to 9% of the human putamen (4-5000 mm^3, as reported in Salvatore *et al*., 2006). This volume is much smaller than those achieved with other infusion methods, such as the use of multiport catheters and CED, and this could therefore explain why the Phase 2 trial (Lang *et al*., 2006) failed.

Main study	Delivery details	Results	Follow up in	Results
Nutt *et al*., 2003 (Phase 1/2)	• right ventricle • monthly injections of GDNF (25, 75, 150, 300, 500 to 4000 µg) or placebo	Serious adverse effects, no improvement	Kordower *et al*., 1999 (analysis of the brain of a deceased patient and of GDNF-injected MPTP-monkeys)	No evidence of nigrostriatal regeneration or of GDNF diffusion to relevant brain regions in the patient
Gill *et al*., 2003 (Phase 1)	• Bilateral (4 patients) or unilateral (1 patient) putaminal continuous delivery via catheter • 14.4 µg/side/day, at 6 µl/hour for 2 months, then 43.2 µg/side/day at 6 µl/hour * * High T2 MRI signal → decrease of the dose back to 14.4 µg/side/day	No serious adverse effects ↑: UPDRS, daily activities, and putaminal dopamine storage ↓: medication-induced dyskinesias	Patel *et al*., 2005 (2-years follow up)	No serious adverse effects ↑: motor and functional performance, Quality-of-Life measures, verbal memory function (3 patients), ^{18}F-DOPA putaminal uptake ↓: medication-induced dyskinesias, verbal memory function (1 patient)
			Love *et al*., 2005 (analysis of the brain of a deceased patient)	↑: TH+ nerve fibres in the putamen Perhaps also increased sprouting of fibres in the SNpc
Lang *et al*., 2006 (Phase 2)	• Bilateral continuous infusions of placebo or GDNF via catheter* • 15 µg/side/day at 6.25 µl/hour * the catheter was smaller than the one used in phase 1	Adverse effects related to the catheter ↑: ^{18}F-DOPA influx constant Development of neutralizing antibodies to liatermin	Salvatore *et al*., 2006 (infusion method)	GDNF injected in rhesus monkeys with the same protocol used by Lang *et al*. was concentrated around the catheter and did not diffuse
			Hutchinson *et al*., 2007 (statistics)	The standard deviation of the results obtained by Lang *et al*. was higher than expected, therefore the study was underpowered
Slevin *et al*., 2005 (Phase 1)	• Unilateral putaminal infusion via multiport catheter • CED-like • Doses increasing at 8-week intervals (3, 10, 30 µg/day)	No serious adverse effects ↑: UPDRS ↓: bradykinesia (all improvements were bilateral)	Trials were halted when Amgen removed r-metHuGDNF from the market ⬇ Slevin *et al*., 2007 (follow up after 1 year without delivery of GDNF)	All the improvements obtained during Phase 1 were lost

Table 2. Summary of PD trials performed with GDNF protein. ↑ indicates an increase, ↓ indicates a decrease. Articles related to the same study have the same background colour.

5.2 Therapeutic potential of virally-delivered NRTN

Research turned to viral delivery of trophic factors, especially *via* AAV2. Viral vectors have several advantages. First, one injection is sufficient to induce expression of the encoded protein for up to several years (Bankiewicz, 2004). Second, the use of viral vectors ensures that the protein is produced in the patient's cells, and it has been shown that, at least in some cases, mammalian-produced neurotrophic factors are of better quality than recombinant factors produced in *E. coli* cells (Hoane *et al*., 2000). Ceregene developed CERE-120, an AAV2-derived vector encoding human NRTN where the prosequence was replaced by that of human NGF in order to improve NRTN secretion (Fjord-Larsen *et al*., 2005). CERE-120 was used in all clinical trials where NRTN was delivered by viral vectors (Table 3). The preclinical study (Gasmi *et al*., 2007) on a rat model of PD showed that NRTN was active and that the protein and some of the vector were retrogradely transported to the SNpc (Kaspar *et al*., 2002, Gasmi *et al*., 2007). After successful trials in a monkey model of PD (Kordower *et al*., 2006), CERE-120 was tested in a Phase 1 clinical trial (Marks *et al*., 2008). In this trial, six patients received low dose of vector ($1.3x10^{11}$ vg) and six a higher dose ($5.4x10^{11}$ vg) injected bilaterally to their putamen: no-one developed serious adverse events, and even if some patients had increased amount of anti-AAV2 antibodies in the serum, this was asymptomatic and the level decreased after six months. After one year of treatment, patients' conditions had improved significantly in both groups, except in one case that was later diagnosed as having multiple system atrophy and not PD (Marks *et al*., 2008).

A Phase 2 trial (Marks *et al*., 2010) was then conducted based on these results. Patients received either $5.4x10^{11}$ vg or sham surgery, and were then monitored every three months for one year, and then followed afterwards, until the last patient had finished the 12-month study. No improvement was seen after one year, but after 18 months there was a significant, although small, difference with the baseline. This delayed response (if compared to the Phase 1 trial) could be probably explained by a worse condition of the patients taking part in the second study. Some serious adverse effects occurred, but they were connected to the surgery procedure, not to the use of the AAV2 vector.

Two patients receiving AAV2-NRTN died of causes unrelated to the ongoing trial, and their brains were analysed to gather more information about the bioavailability of NRTN (Bartus *et al.*, 2010). In this study, the expression of TH and NRTN in those patients was compared to the expression in monkeys (young and aged control monkeys, and an adult monkey model of PD). In both humans and monkeys, NRTN covered 15% of the striatum, but in humans only little NRTN was detectable in the SNpc. This was different from what observed in monkeys: both in aged monkeys and in the PD model, NRTN was detectable also in the nigra, with extensive evidence for retrograde transport. In the same way, TH induction was observed only sparsely in the striata of patients and not in their nigrae, but TH signal was widely present in both structures in monkeys. This discrepancy suggests a difference in the retrograde transport in human PD patients versus animal PD models, and the authors concluded that perhaps targeting directly the SNpc (i.e. the dying neurons) could be more beneficial (Bartus *et al.*, 2010).

Since SNpc is the structure where the somata of the dying neurons are located, one could wonder why this area had not been targeted in any of the clinical trials conducted so far. However,

this had not been done because of safety concerns raised by preclinical studies where GDNF delivered to the nigra had induced robust weight loss in animal models (Zhang *et al*., 1997, Manfredsson *et al*., 2009). Further studies then showed that the weight loss was not due to delivery of the molecule to the nigra, but to its diffusion outside that area. This was due to the viral vector used (AAV5-GDNF), which has lower affinity than AAV2 for the HS in the ECM and therefore diffuses more in the brain. When AAV2-NRTN was delivered adequately, without virus diffusing outside the SNpc, no side effects were observed in rat and monkey models of PD (Bartus *et al*., 2011).

This, and the results of the Phase 2 clinical trial (Marks *et al*., 2010), gave rise to a Phase 1/2 trial, where CERE-120 was delivered both to the striatum and to the nigra of PD patients (Bartus *et al*., 2013). Here, six patients were divided into two groups. Both groups received the same dose of CERE-120 to the nigra ($4.0x10^{11}$ vg), but one received a low striatal dose of the virus ($5.4x10^{11}$ vg, as in the previous Phase 2 trial), while the other was treated with a higher dose ($2.44x10^{12}$ vg), as the lower dose was showed to cover only 15% of the putamen. The dosage for the SNpc was determined by scaling up the dose used for the preclinical studies in rats and monkeys, taking into account the difference in the nigral volume in the different species. The subjects were followed for two years and did not report any serious side effect. This led to a continuation of the study to Phase 2b, but unfortunately there was not a significant difference between the CERE-120 and the sham surgery group. Nevertheless, further data analysis showed that there was a difference in the response to CERE-120 based on how far the disease had progressed: people with a milder disease progress, responded better than those with more advanced PD (Ceregene press release 21.05.2013).

Main study	Delivery details	Results	Follow up in	Results
Marks *et al*., 2008 (Phase 1)	Bilateral intraputaminal CERE-120 ($1.3x10^{11}$ or $5.4x10^{11}$ vg)	No serious adverse effects ↑: UPDRS, time without dyskinesias No increase in ^{18}F-DOPA uptake		
Marks *et al*., 2010 (Phase 2)	Bilateral intraputaminal CERE-120 ($5.4x10^{11}$ vg) or sham surgery	Serious adverse effects No significant differences between patients and controls	Bartus *et al*., 2010 (analysis of the brain of two deceased patients + comparison with PD monkey model)	NRTN covered ~15% of the striatal volume Sparse striatal TH-induction and retrograde transport in patients vs. intense TH-induction and retrograde transport in monkeys
Bartus *et al*., 2013 (Phase 1/2)* * Study planned based on the results of the previous trial	Bilateral intraputaminal ($5.4x10^{11}$ or $2.44x10^{12}$ vg*) and nigral ($4.0x10^{11}$ vg) *The higher striatal dose was used as the other was shown to cover only part of the striatum	No serious adverse effects ↑: UPDRS	Ceregene press release 21.05.2013 (continuation to Phase 2b)	No serious adverse effects No significant difference with placebo-treated controls

Table 3. Summary of trials performed with CERE-120 (AAV2-NRTN). ↑ indicates an increase, ↓ indicates a decrease. Articles related to the same trial have the same background colour.

5.3 Clinical trials of neurotrophic factor delivery in PD: how to improve the results?

With time it became clear that one of the main issues with this type of therapy is the distribution of the neurotrophic factor in the brain. GDNF and NRTN have a high affinity for heparin (Alfano *et al*., 2007) and HS. The ECM is rich in CS and HS (see section 4 and subsections therein) which bind these neurotrophic factors and hinder their diffusion significantly. Indeed, co-infusion of GDNF or NRTN with soluble heparin increases the diffusion volume of these neurotrophic factors (Hamilton *et al*., 2001) and such approach has been patented for GDNF delivery (Rossomando *et al*., 2006). However, this solution is probably not suitable for treating humans, because heparin strongly increases the risk of haemorrhage (Mittal and Rabinstein, 2012). A safer option would be to deliver the neurotrophic factors together with heparin analogues that prevent their binding to the ECM but do not have the same effects of heparin on blood coagulation. A different approach to the diffusion problem would be to deliver GDNF/NRTN mimetics with a lower affinity for HS and which can cross the BBB. A method to screen for such molecules has been patented (Saarma *et al*., 2011).

Another approach is to develop biologically active GDNF (Leung *et al*., 2012) and NRTN variants (Penn *et al*., 2013) with reduced or even absent affinity to heparin. In the case of GDNF, it is known that the part of the sequence which mediates binding to HS is in the N-terminus (see section 2.1 and figure 4), and it is not important for binding to the GDNF receptor (Alfano *et al*., 2007). Therefore Leung and colleagues deleted this region to improve the diffusion and introduced point mutations with different purposes, including reducing immunogenic potential and improving stability. These GDNF variants had an increased diffusion and were active *in vitro* and in *in vivo* models of PD. Their use in a clinical trial was announced in 2011 (Eli Lilly Press Release, 26.04.2011), but no further public information is available. NRTN, instead, does not have such a long N-terminus (see section 2.2 and figure 4). In this case, the residues responsible for binding to HS are in what is predicted to be the heel region. NRTN has not been crystallized, but based on the data about GDNF binding to its receptor, this sequence is not important for binding to the receptor (Parkash *et al*., 2008). Mutation of the heel region could therefore be optimal to improve the diffusion of the molecule (Penn *et al*., 2013).

Another approach could be to use enzymes like Sulf1 and Sulf2 that remove sulphate groups from HS. These groups have been shown to be important for binding of GDNF (Rickard *et al*., 2003), and therefore their removal could improve the diffusion of the molecule and have beneficial effects in PD patients (Ai *et al*., 2007). Studies have shown that HS are important for GDNF signalling *in vitro* (Barnett *et al*., 2002) and for efficient neuroprotection *in vivo* (Piltonen *et al*., 2009). However, the significance of these results is still unclear, since GDNF variants with decreased affinity for heparin are fully active in *in vitro* assays (such as receptor activation and neurite outgrowth) and in *in vivo* 6-OHDA rat models of PD (Alfano *et al*., 2007, Leung *et al*., 2012). Therefore, it is impossible to speculate whether these approaches will be effective.

Lastly, in an effort to combine the advantage of AAV vectors with an increase in the diffusion volume, a new Phase 1 clinical trial recently started. In this trial, 24 patients with PD will receive 4 doses of AAV2-GDNF ($9x10^{10}$ vg, $3x10^{11}$ vg, $9\ x10^{11}$ vg, or $3\ x10^{12}$ vg). To ensure a bigger diffusion volume, the vector will be delivered by CED to the putamen, and patients will be

followed for five years to test the safety and efficacy of this method (ClinicalTrials.gov identifier: NCT01621581).

Diffusion of growth factors is an important issue, but not the only one worth considering. Further analysis of the data obtained in the failed phase 2 AAV2-NRTN trial (Bartus *et al*., 2013) shows that the treatment had actually been effective in patients that had been diagnosed recently with PD. Neurons are post-mitotic cells, and neurotrophic factors cannot stimulate their division in the adult brain. Since neurotrophic factors can only rescue the degenerating neurons, early diagnosis and therapy are essential to treat the disease. Recent studies (Kordower *et al*., 2013) show that within four years from the diagnosis the loss of fibres in the putamen is virtually complete. As it is beneficial to start the therapy as soon as possible to prevent irreversible neuron loss, it would be useful to develop methods to efficiently diagnose the disease during the presymptomatic stage. At the moment research is focusing on tissues, blood and cerebrospinal fluid. Some of the molecules that are being investigated are α-synuclein, homovanillic acid (the most important catabolite of dopamine), markers of oxidative stress, and vitamin D, but at the moment none of these is validated and in use in routine clinical practice (for a complete review, see Saracchi *et al*., 2013).

In addition, patients could benefit from the combination of neurotrophic factor delivery and existing therapies. For instance, it has been shown that exercise increases the levels of BDNF in the serum. BDNF can cross the BBB, and the increased serum levels could therefore have a positive effect on the surviving nigral neurons (Ahlskog, 2011). In addition, BDNF and GDNF have been shown to promote neuron survival *via* different pathways (Onyango *et al*., 2005). Therefore combining exercise with GDNF delivery could have a beneficial, synergistic effect in PD patients.

AIMS OF THE STUDY

The aims of this study were to further characterize the molecular and cell biological properties of GDNF and NRTN, and to study how the modification of their characteristics could beneficially influence their function *in vitro* and *in vivo*.

The specific aims were:

- to characterize how tagging and post-translational modifications of GDNF affect its processing, activity, and stability

- to produce and characterize *in vitro* and *in vivo* NRTN mutants with full biological activity and lower affinity for heparin

- to study the possible effect of reduced heparin binding on internalization and signalling of NRTN and its relevance in clinical settings

MATERIALS AND METHODS

The methods used in this study are presented below. The methods I have used or participated in are marked with a *.

Method	Used in
AKT phosphorylation assay	III
Behavioural assays (cylinder test, amphetamine-induced rotation assay)	II
Binding assays	II
Cell culturing *	I, II
Cell lysates *	I, III
Cell transfection *	I, II, III
Kidney explants	II
ERK phosphorylation assay	III
GFRα affinity chromatography	II
Heparin affinity chromatography *	II (NRTN variants), III (GFRα co-receptors)
Immunocytochemistry *	II
Immunohistochemistry *	II
LI-COR assay	III
Computational modelling of NRTN and its heparin-binding sites	II
N-terminal sequencing	I, II
Partial purification of GDNF *	I
PCR mutagenesis *	I, II
Intracerebral protein injections	II
Protein stability *	I, II
Purification of NRTN *	II
RET phosphorylation assay *	I, II, III
RT-PCR (expression of GFRα1, GFRα2 and RET)	II
Sequence analysis and alignment (Clustal W2 and omega, Expasy prediction tools, ELM)*	III
Diffusion assays *in vivo* (immunohistochemistry and Stereo Investigator platform)*	II
Survival of dopaminergic neurons *in vitro*	II
Protein production	I, II
Western blotting *	I, II, III

RESULTS

6. GDNF: processing, stability, and glycosylation

6.1 Processing of GDNF depends on expression conditions

Adding tags to protein sequences can be useful in many ways. For instance it enables the recognition of exogenously added proteins, allows the use of more sensitive detection techniques, and may make purification easier. However, the choice of tag and insertion site can be crucially important for the folding and activity of the protein. GFLs have seven conserved Cys residues, which are important for the folding of the molecule (Haniu *et al.*, 1996, Eigenbroth and Gerber, 1997). Two of these residues are located at the C-terminus of GDNF and the addition of a tag in this region could be detrimental for the folding of the protein. Therefore we studied how the synthesis and secretion of proGDNF and mature GDNF are affected by N-terminal Flag or His tags. The tags were either directly attached to the protein sequence or linked through two Gly residues. Gly was chosen because of its small size and flexibility (**I**: Table 1).

The His tag impaired the secretion of GDNF, especially in the case of His-tagged proGDNF, but the Flag-tagged molecules seemed to be secreted in high amounts (**I**: Figure 1A). RET phosphorylation assays showed that all the tagged molecules (note that we did not test His-tagged proGDNF because of its poor secretion) are active (**I**: Figure 1D). Due to the secretion issue of His-tagged GDNF, we focused on further characterization of the Flag-tagged Gly-linked proteins (proFlagGGGDNF and FlagGGGDNF). CHO or HEK 293 cells were transfected with plasmids encoding ProFlagGGGDNF and FlagGGGDNF, and the media were collected one and two days later and analysed. The amount of the protein in the medium increases with time, and there is a difference between the cell lines, as proGDNF might be processed more in HEK 293 cells (**I**: Figure 1B). These results also show that mature GDNF can be secreted without the prosequence, at least when overexpressed.

We also compared the effects of different media on the processing of GDNF. We transfected CHO cells with plasmids encoding untagged proGDNF, ProFlagGGGDNF or FlagGGGDNF and grew them either in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin or OptiMEM® without FBS. We then collected and analysed media and lysates: in the presence of OptiMEM® the secretion of GDNF is less efficient (**I**: Figure 1C).

As GDNF processing pattern is quite complex and gives rise to several bands visible on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, we decided to sequence part of them to verify their identity. GDNF was partially purified, run on a reducing gel and then transferred on a PVDF membrane. We detected a difference in the band pattern. ProGDNF generated several bands, one of which was replaced by two bands when the ProFlagGGGDNF was analysed (**I**: Figure 2). We sequenced these two bands, and found that the heavier band corresponded to FlagGGGDNF (DYKDDDDKGGSPDKQMAVLP…), while the lighter band lacked the FlagGG and the first six amino acid residues of the sequence of mature GDNF (AVLP…). The single band generated by proGDNF was *de facto* a mixture of normal length mature GDNF (SPDKQMAVLP…) and shorter GDNF (AVLP…), showing that the novel cleavage site is not caused by the presence of the tag. We tried to study whether it was possible to

inhibit this cleavage by mutating one, two, or three amino acids located in this region (SPDKQM|AV → SPDKQ**A**|AV, SPDK**AA**|AV, or SPD**AAA**|AV, | indicates the cleavage site). However, we did not detect any significant difference between the constructs (data not shown).

6.2 GDNF is more stable when produced in mammalian cells

GFLs are conserved among different species, but are not produced by bacteria in nature. However, it is possible to transform *E. coli* with plasmids encoding GDNF in order to produce large amounts of growth factor. This method has been used in order to produce GDNF for clinical trials (listed in Table 2), but mammalian GDNF is known to be glycosylated (Lin *et al*., 1993, Ansorena *et al*., 2010) and the disulphide bonds present in the GDNF are fundamental for its function (Eigenbroth and Gerber, 1997). Neither of these modifications occurs in *E. coli*. In addition, the purification and subsequent *in vitro* renaturation of the molecule in general occur through a series of harsh steps (Lin *et al*., 1993, Hoane *et al*., 2000). The renaturation step is crucial to obtain functional growth factors with disulphide bonds, but it probably forms proteins with slightly altered tertiary structure, and subsequently reduced biological activity (Hoane *et al*., 2000). We therefore wanted to assess whether production of GDNF in a more natural environment would be beneficial for the stability and/or activity of the molecule.

We transfected CHO cells with green fluorescent protein (GFP), tagged or untagged proGDNF, and collected the media two days later. *E. coli* GDNF was added to the medium from GFP-transfected cells, and all the media were subsequently incubated at 37 ºC in the absence of cells. Results showed that *E. coli* GDNF is less stable than mammalian GDNF, regardless of the tag (**I**: Figure 4A). Stability of GDNF varies depending on the extracellular environment, and different cell types process the molecule differently (**I**: Figure 4C). Regarding the activity we performed RET phosphorylation assays on MG87-RET cells transfected with GFRα1 without detecting any difference in the strength or duration of the phosphorylation induced by either mammalian or *E. coli* GDNF (data not shown), but it has to be taken into account that this setting may not be sensitive enough.

6.3 Glycosylation affects maturation of GDNF but not its receptor specificity

GDNF is glycosylated (Lin *et al*., 1993), however, no studies have investigated where this modification specifically occurs and what its role is. First of all we decided to align the sequences of GDNF from several species to figure out whether this modification is conserved (Figure 8), and then to compare the sequences of human GFLs to verify whether the other members of the same family are also glycosylated (Figure 4).

```
G. GALLUS       ------------------------------LWDVVAVCVVLLNTVSTSPLPAGKMPPEGPPS  32
N. NIPPON       ------------------------------------------------------------  0
A. MELANOLEUCA  ------------------------------------------------------------  0
B. TAURUS       ------------------------------------------------------------  0
M. MUSCULUS     MGFGPLGVNVQLGVYGDRIRGAAAGRDSKMKLWDVVAVCLVLLHTASAFPLPAGKRLLE----  59
R. NORVEGICUS   ------------------------------MKLWDVVAVCLVLLHTASAFPLPAGKRLLE----  30
H. SAPIENS      ------------------------------MKLWDVVAVCLVLLHTASAFPLPAGKRPPE----  30
M. MULATTA      ------------------------------MKLWDVVAVCLVLLHTASAFPLPAGKRPPE----  30
X. LAEVIS       ------------------------------MKLWAILAVCILLLSSVSSIPLPSNWLAGKKRSH  34
D. RERIO        ------------------------------MKLWDILATCLLLLSSVSTRPLFHKLQPSKRAVV  34
C. CARPIO       ------------------------------------------------------------  0

G. GALLUS       VVEGPED------------DLSPI-SLPPPYAVHSDSNMPEDYPDQFDEVVDFIQATIKRLRR  82
N. NIPPON       ------------------------------------------------------QATIKRLRR   9
A. MELANOLEUCA  ------------------------------------------------------QATIKRLKR   9
B. TAURUS       ------------------------------------------------------------  0
M. MUSCULUS     ---APAE------------DHSLG-HRRVPFALTSDSNMPEDYPDQFDDVMDFIQATIKRLKR 106
R. NORVEGICUS   ---APAE------------DHSLG-HRRVPFALTSDSNMPEDYPDQFDDVMDFIQATIKRLKR  77
H. SAPIENS      ---APAE------------DRSLG-RRRAPFALSSDSNMPEDYPDQFDDVMDFIQATIKRLKR  77
M. MULATTA      ---APAE------------DRSLG-RRRAPFALSSDSNMPEDYPDQFDDVMDFIQATIKRLKR  77
X. LAEVIS       LP-DPQEGEDQVFGMDGAVPEDPT-ANMAPQDQQTYTEIPDDYPDQFDDVLEFIQDTIKRLKR  95
D. RERIO        RSESPAL--------DPIIDSQPETSNPKQASMEEQYDLTGLYPEQFEDVMDFIEATLGRLRR  89
C. CARPIO       ------------------------------------------------------------  0

G. GALLUS       SPDKQTPIFSRR-ERNRQSAATNVENSSKKGRRN---------------QKGKNRGCVLTAIHL 130
N. NIPPON       SPDKQTPIFSRR-ERNRQNAATNIENSSKKGRRN---------------QKGKNRGCVLTEIHL  57
A. MELANOLEUCA  SPEKQVAVPPRR-ERNRQAAAGSPENARGKGRRG---------------QRGRNRGCVLTAVHL  57
B. TAURUS       ----------PRR-ERHRQGAAASPESARGKGRRG---------------QRGRNRGCVLTAVHL  39
M. MUSCULUS     SPDKQAAALPRR-ERNRQAAAASPENSRGKGRRG---------------QRGKNRGCVLTAIHL 154
R. NORVEGICUS   SPDKQAAALPRR-ERNRQAAAASPENSRGKGRRG---------------QRGKNRGCVLTAIHL 125
H. SAPIENS      SPDKQMAVLPRR-ERNRQAAAANPENSRGKGRRG---------------QRGKNRGCVLTAIHL 125
M. MULATTA      SPDKQMAVLPRR-ERNRQAAAANPENSKGKGRRG---------------QRGKNRGCVLTAIHL 125
X. LAEVIS       SSNKQPPS---RRDRGRQSLAANTQISSKKTVKD---------------RKRKNKGCVLREIHL 141
D. RERIO        SSDVEPQM---KRDRVRQKAAANTEKSGGRGRGERKRSRGRARSRDDRVKGQGRGCLLKEIHL 149
C. CARPIO       ------------------------------------RGRKESRDDRVKGQGRGCLLKEIHL  25
                                                                  ** *   **

G. GALLUS       NVTDLDLGYETKEELIFRYCSGSCDAVETTYDKILKNLTRKKKLVNDKVRQACCRPTAFDDDL 193
N. NIPPON       NVTDLDLGYETKEELIFRYCSGSCDAAETTYDKILKNLTRKKKLVTDKVRQACCRPTAFDDDL 120
A. MELANOLEUCA  NVTDLGLGYETKEELIFRYCSGSCDAAETMYDKILKNLSKNRRLVSDKVGQACCRPIAYDDDL 120
B. TAURUS       NVTDLGLGYETKEELIFRYCSGSCDAAETMYDKILKNLSKSRRLVSDKVGQACCRPIAFDDDL 102
M. MUSCULUS     NVTDLGLGYETKEELIFRYCSGSCESAETMYDKILKNLSRSRRLTSDKVGQACCRPVAFDDDL 217
R. NORVEGICUS   NVTDLGLGYETKEELIFRYCSGSCEAAETMYDKILKNLSRSRRLTSDKVGQACCRPVAFDDDL 188
H. SAPIENS      NVTDLGLGYETKEELIFRYCSGSCDAAETTYDKILKNLSRNRRLVSDKVGQACCRPIAFDDDL 188
M. MULATTA      NVTDLGLGYETKEELIFRYCSGSCDAAETTYDKILKNLSRNRRLV----------PTAFDDDL 178
X. LAEVIS       NVTDLGLGYETKEELKFRYCSGSCNNPETTYDQILKNLTIRKKLVNDKVKQACCRPIAFDDDL 204
D. RERIO        NVTDLDLGYRTKEELIFRYCSGPCHDAETNYDKILNNLTHNKKLDKDTPSRTCCRPIAFDDDI 212
C. CARPIO       NVTDLGLGYRTKEELIFRYCSGPCFDAETNYDKILNNLTHNKKLDKETPSRTCCRPVAFDDDI  88
                ***** *** ***** ****** *   ** ** ** **     *          * * ***

G. GALLUS       SFLDDNLVYHILKKHSAKRCGC- 215
N. NIPPON       SFLDDNLVYHILKKHSAKRCGCI 143
A. MELANOLEUCA  SFLDDNLVYHILRKHSAKRCGCI 143
B. TAURUS       SFLDDNLVYHILRKHSAKR---- 121
M. MUSCULUS     SFLDDNLVYHILRKHSAKRCGCI 240
R. NORVEGICUS   SFLDDSLVYHILRKHSAKRCGCI 211
H. SAPIENS      SFLDDNLVYHILRKHSAKRCGCI 211
M. MULATTA      SFLDDNLVYHILRKHSAKRCGCI 201
X. LAEVIS       SFLDDNLVYHTLKQHSAKKCGCI 227
D. RERIO        SFLDDSLEYHTLKKHSAKKCACV 235
C. CARPIO       SFLDD------------------ 93
                *****
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Figure 8. Alignment of GDNF from different species. Red: presequence, light blue: prosequence, green: finger structures, **C**: cysteines involved in the knot, NXX: putative N-glycosylation sites, the red vertical bar indicates a predicted furin cleavage site. The sequences of GDNF (or fragments of it) from different species were aligned and analysed as described in Figure 4.

The predictions point out that GDNF has two putative N-linked glycosylation sites (three in birds: *G. gallus* and *N. nippon*), which are conserved among birds, mammals, amphibians, and fish.

We mutated the predicted glycosylation sites of human GDNF and expressed the protein in the presence of tunicamycin, which inhibits N-linked glycosylation. Results show that only the site located on the first finger (126NVT) is in use (**I**: Figure 3), at least in the cell line used (Chinese hamster ovary cells, CHO). In our set up, glycosylation at this site is not important for receptor specificity (at least in the presence of high amounts of GDNF) or stability of the molecule (**I**: Figure 4B). However, we show that glycosylation at this site affects the processing and secretion of GDNF: the amount of growth factor in the medium is lower, and it seems to be secreted mostly as proGDNF (**I**: Figure 3 and 4B).

7. NRTN mutants: production, testing, and role of heparin binding

7.1 Production and purification of the NRTN variants

To create NRTN mutants, we decided to model the three-dimensional structure of NRTN (**II**: Figure 1A) based on homology with GDNF, one of the only two members of the family that have been crystallized (Eigenbroth and Gerber, 1997, Parkash *et al*., 2008). This was necessary in order to find the residues that could be involved in binding to heparin: the amino acids not only have to be part of a specific consensus sequence (Cardin and Weintraub, 1989), but they have to be aligned so that they point in the same direction. The heparin-binding residues are located in the helix, while studies on GFLs have shown that the regions important for binding to the receptor are in the fingers (Eketjäll *et al*., 1999, Baloh *et al*., 2000, Parkash *et al*., 2008). Therefore we thought that mutations in this area would not have a major effect on receptor binding. The helix region is quite long and comprises several basic Arg residues, with more than one potential heparin-binding consensus sequence. For this reason, and because of the risk of generating non-functional mutants, we decided to produce more than one variant (**II**: Figure 1B). The first three mutants (N1-N3) were planned based on the replacement of Arg with Ala in the helix region: N1 and N2 have non-overlapping mutations, while in N3 we combined the mutations present in both N1 and N2, with the exception of the last Arg. This choice was made because this residue is conserved in three out of four GFLs (Figure 4) and could therefore be important. The fourth variant (N4) was engineered by exchanging the helix of NRTN with that of PSPN and adding four extra residues to maintain the length of the helix. The reason for this choice is that PSPN does not bind heparin (Bespalov *et al*., 2011). For initial use we also produced N-terminally V5-tagged variants (NV1-4) in order to be able to detect the proteins with high sensitivity. In addition, to improve secretion, we cloned NRTN variants lacking the prosequence and harbouring the immunoglobulin G signal sequence (Fjord-Larsen *et al*., 2005).

We transfected CHO cells with plasmids encoding these mutants and verified the decreased affinity for heparin by affinity chromatography. All the variants presented decreased heparin affinity (**II**: Figure S1C). RET phosphorylation experiments with media from CHO cells transfected with plasmids encoding the NRTN variants showed that all the variants were active (**II**: Figure S1A-B), so we proceeded with their purification and functional testing.

To upscale NRTN production, the variants (without V5-tag and prosequence) were produced in CHO cells in suspension. We chose mammalian cells for NRTN expression to ensure a better quality of the molecule (Hoane *et al*., 2000).

7.2 The NRTN variants have reduced affinity to heparin and two of them are more active than wild type NRTN *in vitro*

As the NRTN variants had decreased affinity for heparin, and different pI values, the established purification techniques for GFLs could not be used. We therefore developed a new method based on a first partial purification with a heparin column, and a second step based on affinity to GFRα2. After this procedure, we obtained relatively pure proteins (**II**: Figure 2); however, a small fraction of wild type NRTN and N2 lacked two amino acids at the N-terminus.

Heparin affinity chromatography shows a decreased binding to heparin of the purified untagged NRTN variants (**II**: Figure 3, wild type NRTN eluted at 1.08 M NaCl, N1 at 0.97 M, N2 at N3 at 0.56 M, N4 at 0.48 M). Our decision to produce several mutants proved to be very good, as during heparin affinity chromatography assays N1 was really difficult to handle. In addition, when later the variants had to be concentrated for *in vivo* assays, both N1 and N3 had aggregation and precipitation problems. We can speculate that these problems are due to the mutations they share, and that charges normally present in this area prevent NRTN molecules from aggregation. However, it must be taken into account that also wild type NRTN has a strong tendency to aggregate and precipitate.

The *in vitro* assays gave very interesting results. Immunocytochemical stainings (**II**: Figure 6A-B) show that when untransfected CHO cells are incubated with wild type NRTN, the molecule binds to the HSPGs present on the cell surface, while N4 fails to do so. However, when the cells are transfected with GFRα2, both variants can bind the receptor. Immunocytochemistry is not quantitative, so we decided to perform binding assays to verify whether there was a difference in the affinity for the receptor and the strength of the binding. Our binding assays performed on affinity columns show that binding of the NRTN variants to GFRα1 is weaker than to GFRα2 (**II**: Figure S4). This is in line with what is known about wild type NRTN (Cik *et al*., 2000). On the affinity columns, in the absence of RET, the mutants seem to bind slightly more weakly to GFRα2 than wild type NRTN (**II**: Figure S4). The difference in the binding to GFRα2 is however abolished when RET is present on transfected cells (**II**: Figure 4A), in line with the fact that the receptor stabilizes the complex (Virtanen *et al*., 2005). We conclude that probably the mutation introduced altered slightly the orientation of the finger-like structures of NRTN, which are responsible for receptor binding. However, even if such a change had happened, its magnitude was not enough to have a significant effect on the binding of NRTN to its receptor.

Once proved that the binding to GFRα2 and to GFRα2/RET was almost unaltered, we proceeded with several activity assays. There is apparently no difference in the doses at which wild type NRTN or the variants activate RET (**II**: Figure 4B) or promote survival of embryonic dopaminergic neurons (**II**: Figure 5A). This alone, would indicate that there is no superiority of any of the variants compared to the wild type protein. However, when the system where the molecules are tested gets more complex, N2 and N4 prove to be more efficient than wild type NRTN. One such example is the embryonic kidney explant culture (**II**: Figure 5B): NRTN is known to

work as autocrine factor in the kidney branching *in vitro*, but it does not induce extraureteric branching (Davies *et al*., 1999). This effect, however, is known to occur in the presence of GDNF (Sainio *et al*., 1997). Our variants N2 and N4, instead, promote such branching, probably because of their high stability. Western blots with samples taken from the media where the kidney explants were growing show that, while wild type NRTN is degraded, N2 and N4 are present even after 24 h (**II**: Figure 5B). The reason for the higher stability of N2 and N4 is not clear yet. However our studies suggest the involvement of an unknown protease which could cleave wild type NRTN but not the variants because of the mutations introduced (**II**: Figure S2B-D). This improved stability has of course beneficial effects even in clinical use of NRTN, when the molecule could act longer on the degenerating and dying neurons.

7.3 N4 improves neurorestoration and motor function in a rat 6-OHDA model of PD

We injected wild type NRTN, N2, and N4 in the brains of naïve rats, measured the diffusion volumes, and showed that N4 is the one that diffuses the most (**II**: Figure 6C-D). A similar diffusion was observed in the brain of two different monkey species (**II**: Figures S5 and S6), showing that the improved diffusion properties are maintained in other species.

The benefits of the improved stability and diffusion are observed in *in vivo* experiments on rat models of PD. In this experiment we decided to compare the variants to GDNF produced in *E. coli*, which is more effective than NRTN in animal models of PD and has been used in all the GDNF-related clinical studies. We decided to administer a relatively high dose of 6-OHDA (28 µg 6-OHDA distributed to four striatal injection sites), followed by low amounts (5 µg) of neurotrophic factors. The neurotrophic factors were administered intrastriatally, two weeks after the lesion. This way we were able to compare the effects of the different treatments and see which one was the most effective. With a smaller lesion and saturating amounts of factors, we might not have been able to compare their activities. We tested the use of paws (cylinder test, **II:** Figure 7C) and measured amphetamine-induced rotations (rotation assay, **II**: Figure 7B). Normally animals use both their paws and do not rotate when administered amphetamine. A unilateral lesion of dopaminergic neurons, however, causes the animals to use preferentially the ipsilateral limb and to rotate toward the lesion side. If the administered neurotrophic factors rescue the dying cells, unilateral limb use deficiency gets smaller, and the number of rotations decreases. Of all the administered molecules (*E. coli* GDNF, N2, N4, and buffer), only N4 had a significant effect in both tests. When the brains of the animals were analysed, we observed that all the neurotrophic factors used rescued the dopaminergic somata (**II**: Figure 7D), but only in animals treated with N4 also the axons were preserved (**II**: Figure 7E). GDNF and the two NRTN variants N2 and N4 have also been tested in monkey MPTP models of PD to measure their neurorestorative activity. The results are promising, and are currently being analysed by our collaborators. They will form an article not included in this thesis.

The NRTN variants, especially N4, have proved to be effective in a rat animal model of PD. However, in order to use them successfully in humans it is important to verify whether the component of the receptor complex are expressed in the brain regions of interest. We therefore verified using RT-PCR on a human brain sample that the mRNAs of *Gfrα1*, *Gfrα2* and *Ret* are expressed in the striatum and in the SNpc (**II**: Figure 8B).

7.4 Binding to heparin causes increased accumulation of NRTN and earlier onset of RET signalling

To verify that the decreased binding to heparin would not affect other important properties and to understand its potential importance, we compared accumulation, internalization and signalling of wild type NRTN and of NV4.

Results show that the presence of HS, either on the cell surface or in soluble form in the medium, aids the accumulation of wild type NRTN on the cell surface. This is true not only in the absence of GFRα2 (**II**: Figure 6A-B), but also when GFRα2 (alone or together with RET) is present (**III**: Figures 3 and 4). The higher amount of wild type NRTN can be detected both at 37ºC and on ice, when the endocytosis is blocked. This indicates that the effect on the accumulation starts already at the cell surface level.

We studied whether NRTN is internalized in the absence of RET. We performed assays using the LI-COR system, and CHO cells transfected with GFRα2 together or without RET, or untransfected cells (**III**: Figure 5). Our results show that wild type NRTN can bind the surface of CHO cells lacking either receptor and is subsequently internalized, while NV4 fails to do so. When GFRα2 is present, however, both variants bind it and are internalized, independently of the presence of RET. The total amount of internalized NRTN in the presence of RET seems however to be lower than when only GFRα2 is present. This could be due to increased degradation or to lower amounts of GFRα2 on the cell surface. Further experiments are needed to answer this question.

As the increased accumulation of wild type NRTN could have an effect on signalling, we decided to measure phosphorylation of RET, AKT and ERK in different cell lines (**III**: Figure 6). First, we transfected MG87-RET cells (which express RET) with GFRα2, and incubated them at 37 ºC with wild type NRTN or NV4 for different amounts of time. Results show no difference between the NRTN variants (**III**: Figure 6A). Then we repeated the experiment in CHO and pgsA 745 cells transfected with both RET and GFRα2. These cell lines were chosen to study the effects of the presence of HS, as pgsA 745 is a mutant CHO cell line which does not produce HS. Also in this case there was no difference between the NRTN variants (**III**: Figure 6B). However, when we used Neuro 2A cells (which express both RET and GFRα2 at low endogenous levels), we detected a difference between wild type NRTN and NV4. Results show that wild type NRTN induces phosphorylation of RET already after a short incubation of 1 min, while NV4 fails to do so (**III**: Figure 6C). Therefore, our results indicate a correlation between a stronger affinity to heparin and a faster signal onset when the components of the recptor complex are expressed at endogenous levels. As GFRα2 has relatively high affinity to heparin (**III**: Figure 1) this faster onset could for instance be due to HSPGs acting as a link between NRTN and the co-receptor.

DISCUSSION

8. GDNF processing, stability, and glycosylation

We show that the tagging, the cell line and the medium used can affect the processing of GDNF. This is critically important to take into account when producing proteins for clinical use, as the different processing of GDNF can result in different efficacy of the growth factor. Another thing to consider is the presence of a shorter form of mature GDNF, which lacks six residues at the N-terminus and is produced at least during overexpression of the growth factor. One could argue that this is not important because the binding to GFRα1 occurs through the fingers (Baloh *et al*., 2000), and the residues lacking in the shorter form of GDNF are not part of the heparin-binding sequence (Alfano *et al*., 2007). However, tagging can be beneficial in experimental settings order to recognize exogenous protein, and for purification. Therefore it is worth to consider this additional cleavage site when planning to N-terminally tag GDNF for experimental studies.

The r-metHuGDNF used in clinical trials (Table 2) was produced in *E. coli* as mature protein. The possibility to obtain the same molecule from mammalian cells without the need to use harsh purification and renaturation steps is a very good alternative, especially when the goal is to produce high-quality GDNF for clinical use. We show that GDNF can be secreted from mammalian cells also when overexpressed without the prosequence. However, the prosequence of GDNF may be active on its own. Indeed, it has been shown that DNSP-11, a peptide composed of 11 amino acids belonging to GDNF prosequence, has neuroprotective effects on dopaminergic neurons (Bradley *et al*., 2010). Due to low heparin affinity, DSNP-11 likely diffuses more than GDNF in the brain tissue (Kelps *et al*., 2011). It is not known if this peptide is naturally cleaved from proGDNF, but if this was the case use of proGDNF could lead to unexpected side effects due to DSNP-11 activity and diffusion. On the other hand, the presence of the prosequence in expression constructs for mammalian cells seems to increase the amount of secreted pro- and mature GDNF. The prosequence could therefore be important, especially when delivering GDNF intracerebrally through viruses or encapsulated cells. It must also be taken into account that the two isoforms of GDNF (α-proGDNF and β-proGDNF) differ only in their prosequence but are differently secreted, as β-proGDNF, but not α-proGDNF, secretion is activity-dependent (Lonka-Nevalaita *et al*., 2010). Therefore whether to include or not the prosequence and which one to choose might depend on the specific goals of the experiment or clinical trial.

As mentioned, GDNF produced in *E. coli* is purified through several steps, including renaturation to reform disulphide bonds, which could affect its quality. In addition, since bacteria do not naturally N-glycosylate molecules or produce disulphide bridges, the molecule obtained is inevitably different from the mammalian one, even when cells are transformed with plasmids encoding the human sequence. However, clinical trials have so far been conducted with GDNF produced in *E. coli*. Because of possible differences due to the production system we wanted to verify whether the quality of GDNF produced in mammalian cells is functionally comparable or even superior to that produced in *E. coli*. Using RET phosphorylation assay we could not detect any differences in the activity of GDNF from *E. coli* or mammalian cells, but this could be due to the assay conditions. The experiments were in fact performed with excess GDNF amounts and

overexpression of the receptor complex and therefore the experimental conditions might not be sensitive enough to show possible differences (see also NRTN discussion).

What we show, however, is that *E. coli* GDNF is less stable than mammalian GDNF. This could be due to the way the molecule is purified, to not correctly formed Cys bridges or to lack of glycosylation. We decided to study the last question in more detail. Human GDNF is predicted to be glycosylated close to the tip of the first finger and on the α-helix, just before a putative furin cleavage site. As the fingers are important for binding to GFRα1 (Eketjäll *et al*., 1999, Baloh *et al*., 2000, Parkash *et al*., 2008) glycosylation on the first predicted site could play a role in receptor specificity. Glycosylation on the second site, instead, could protect GDNF from degradation, by making the predicted neighbouring furin cleavage site inaccessible to proteases (Figure 8). Our results indicate that only the first site is N-glycosylated (at least in the cell line used), and this is important for the processing and secretion of GDNF. Specifically, GDNF lacking glycosylation is found in the medium mostly as proGDNF, while the mature unglycosylated mammalian protein is secreted only in very low amounts. However, both unglycosylated mammalian proGDNF and unglycosylated mammalian GDNF are more stable than *E. coli* GDNF. This suggests that the lower stability of bacterially-produced GDNF is not due to absence of glycosylation but rather to suboptimal folding.

Taken together, these results suggest a role of glycosylation in processing and secretion of GDNF, and show that mammalian GDNF is more stable than the one produced in *E. coli*. Compared to *in vitro* assays (1-100 ng/ml, see for instance Lin *et al*., 1993, Eigenbroth and Gerber, 1997, Baloh *et al*., 2000), quite high concentrations of GDNF (3-100 µg in injection experiments, 3-10 µg/day in chronic infusion) were used in *in vivo* experiments (see for instance Hoffer *et al*., 1994, Kirik *et al*., 2001, Piltonen *et al*., 2009) and in clinical trials (3-43 µg/day, see Table 2). The need for higher doses could be due to a differential degradation of GDNF in the brain tissue (Hadaczek *et al*., 2010), to aggregation and subsequent precipitation, and/or to a non-optimal quality of the protein. This last hypothesis is supported by the fact that whenever mammalian GDNF has been used in animal models, much lower doses were sufficient to promote survival of dopaminergic neurons. As an example, in AAV-GDNF treated animals (Kirik *et al*., 2000) 0.22-2.28 ng GDNF/mg tissue was found in the injected tissue, and when animals were transplanted encapsulated cells the initial secretion of these capsules was 26.4-67.9 ng/day (Kishima *et al*., 2004, Sajadi *et al*., 2006). In some animal experiments (Kirik *et al*., 2001) continuous injections of *E. coli*-produced GDNF have been used. Therefore the difference in the doses cannot be explained by a different experimental paradigm (single protein injection versus continuous cell secretion).

9. Affinity of NRTN to heparin: two sides of the same coin

Taking all our results into account, we conclude that the improved diffusion and stability of the N4 NRTN variant are important for its higher efficacy compared to wild type NRTN and GDNF. N4 is more active than wild type NRTN in promoting extraureteric budding in kidney explants, and more effective than GDNF in restoring dopaminergic fibres and motor function in a rat model of PD. Another factor that could account for the improved activity of N4 compared to *E. coli* GDNF could also be the production of the NRTN variant in mammalian cells. The im-

proved diffusion and stability probably affect the activity of N4 so that this variant reaches a higher number of neurons and induces survival or regeneration of their axons and not only of their somata. The effect on axons is reflected in the results of the behavioural assays.

HS and CS are essential components of the ECM, but their exact composition is most likely different in different species (see for instance Lu *et al*., 2010). However, we show that N4 diffuses more than wild type NRTN in rats, and more than GDNF in two different monkey species (cynomologus and marmoset monkeys). If the improved diffusion feature is conserved in humans, treatment with N4 could be more beneficial than treatment with *E. coli*-produced GDNF or AAV2-NRTN.

On the other hand, we report that the presence of HSPGs on the cell surface or in the medium aids the accumulation of wild type NRTN, but does not affect that of N4. The increased accumulation of wild type NRTN results in a more effective signalling in Neuro 2A cells. This cell line expresses GFRα2 and RET endogenously, and therefore at lower levels than transfected CHO, pgsA 745 or MG87-RET cells: this is perhaps the reason why the assay is more sensitive and the difference between the variants can be measured. One could wonder whether this difference in the amount of factor on the cell surface should not affect the activity of the proteins injected in the brain so that N4 would be less active than wild type NRTN. We have not used wild type NRTN in our *in vivo* setup, but we can speculate that, as GDNF is also binding HSPGs, the presence of HSPGs could affect the accumulation of GDNF (and therefore its activity) similarly to what happens with wild type NRTN. However, in the *in vitro* activity assay N4-containing medium was replaced with DMEM after 1 min, while when injected into the brain the molecule is continuously present and has therefore more time to act. This way, the only apparent deficit of N4 is overcome and the molecule proves to be superior to GDNF for *in vivo* use.

Similar mutagenetic approaches might be useful to improve the diffusion of the other GFLs (of course with the exception of PSPN). As a matter of fact, GDNF variants with lower affinity to heparin have already been patented (Leung *et al*., 2012). However, it is extremely important to plan the mutation carefully: we modelled the three-dimensional structure of NRTN to study which amino acid residues were correctly aligned in space, but in other GFLs the heparin-binding region can be localized somewhere else. In GDNF, for instance, the N-terminus is important for binding to heparin (Alfano *et al*., 2007), but the helix contains only a few basic residues. In that case, replacing the α-helix with that of PSPN would have failed to generate mutants with increased diffusion. In the same way, to generate ARTN variants, the exact sequence and structure have to be considered, and only after a careful analysis the mutation(s) can be planned. Ideally, they should not be located on the fingers, as mutation of these structures could affect receptor binding, and mutations with positive effects on the stability could also be desirable. In our case, the residues important for heparin binding are located in the heel region (Figure 4, residues 146-158), so their mutation does not significantly affect the interaction with the receptors. In addition, introduction of the helix of PSPN improves the stability of NRTN, as N4 is more stable than the wild type protein.

Another way to improve the diffusion of neurotrophic factor could be the use of different types of viral vectors. AAV5 viruses have lower affinity to heparin than AAV2 viruses (Opie *et al*., 2003), and could therefore diffuse better once delivered to the brain. However, this type of

therapy would require better titration, as diffusion of AAV5-GDNF outside the SNpc has been shown to cause robust weight loss in animal models (Manfredsson *et al*., 2009). In addition, the use of AAV vectors does not allow precise regulation of the amount of neurotrophic factor. Therefore at the actual state, delivery of purified mammalian protein might still be preferable.

We also show that both wild type NRTN and N4 can be internalized when GFRα2 but not RET is present. There is therefore a RET-independent internalization pathway, which could be related to the interactions with N-CAM (Paratcha *et al*., 2003), but this still has to be analysed in more details. A similar conclusion can be made for GFRα2-independent NRTN internalization, since we show that wild type NRTN can be internalized even in the absence of this receptor if there are HS on the cell surface. This internalization could be caused by interactions with HSPGs (Bespalov *et al*., 2011), but, again, more studies have to be performed. The significance of this result in *in vivo* context is still to be determined.

The use of NRTN in PD clinical trials is of course useful only if RET and GFRα1 and/or GFRα2 are expressed in the relevant areas of the brain (striatum and/or SNpc). Studies on GFRα1 knock-out E12 mouse embryos (Cacalano *et al*., 1998) show that their dopaminergic neurons do not benefit from the presence of GDNF of NRTN, unless also exogenous GFRα1 (Cacalano *et al*., 1998) or GFRα2 (Wang *et al*., 2000) are added. These results lead to the important conclusion that both GDNF and NRTN can promote dopaminergic neuron survival, if either GFRα1 or GFRα2 is present. Considering this, the failure to rescue dopaminergic neurons in GFRα1 knock-outs suggests that GFRα2 is not present on these neurons in new born mice, or at least its levels are too low to mediate an effective signalling. This is in accordance with *in situ* hybridization data which show that in developing and adult mice (Widenfalk *et al*., 1997, Wang *et al*., 2000) and in adult rats (Marco *et al*., 2002) *Gfrα1* mRNA is expressed by the nigral dopaminergic neurons, but *Gfrα2* mRNA is mostly expressed by neighbouring cells. Only a subpopulation of dopaminergic neurons located in the deep midbrain expresses low levels of *Gfrα2* mRNA. These observations generate the conclusion that, at least in mice models of PD, the rescuing activity of GDNF and NRTN is mediated by GFRα1. In our study we show that, in adult humans, mRNAs encoding GFRα1, GFRα2 and RET are present both in the striatum and in the nigra (**II**: Figure 8B). However, we performed PCR on tissue samples which contained several cell types, and therefore our results do not specifically demonstrate the expression of GFRα1-2 or RET in neurons (or dopaminergic neurons). There can also be developmental differences and humans, mice and rats might have a different expression pattern of the receptor. Walker and colleagues (1998) show that *Ret* and *Gfrα1* mRNAs are expressed in the SNpc of PD patients. The levels of *Ret* mRNA vary between samples. The authors mention that they cannot make conclusions about the biological relevance of this finding because it is not known what levels of RET are required to confer responsiveness to GDNF. Moreover, they do not comment on the stage of PD, and their analysis of the expression of *Gfrα1* mRNA is based on one patient only. Bäckman *et al* (2006) measured the expression of *Gdnf*, *Gfrα1* and *Ret* mRNAs in the putamen of PD cases, and showed that the expression levels of the components of the receptor complex are unchanged in PD patients compared to age-matched controls. Taken all this into account, and considering that NRTN can signal through GFRα1, there are good reasons to think that our NRTN variants with improved diffusion might have a positive effect on PD patients.

CONCLUSIONS

This thesis was conducted to obtain better understanding on the post-translational modifications of GDNF and on the heparin binding feature of NRTN. These studies have interesting implication for clinical use of GDNF and NRTN.

The main conclusions from this work are:

I. Glycosylation on the first finger of GDNF does not affect stability of the growth factor or its receptor specificity.

II. GDNF produced in mammalian cells is more stable than GDNF produced in *E. coli*.

III. The affinity of NRTN to heparin is correlated to a more effective onset of intracellular signalling.

IV. The helix of NRTN can be modified without affecting significantly the interaction of the growth factor with the receptor. The mutation increases diffusion and stability of the growth factor.

V. The increased diffusion and stability lead to higher efficacy of NRTN in a rat 6-OHDA model of Parkinson's disease.

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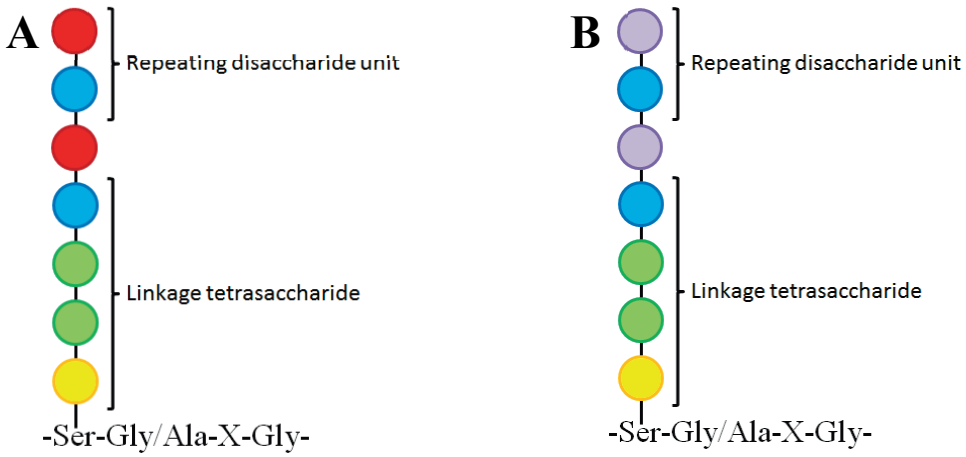

Figure 7. A. Synthesis of CS. In dermatan sulphate, the GlcA of the repeating disaccharide unit is replaced by IdoA. **B. Synthesis of HS.** Yellow: Xyl, green: Gal, light blue: GlcA, red: GalNAc, lilac: GlcNAc.

The sugar structure is then sulphated: CS have long tracts consisting of modified disaccharides. Some types of sulphation are present both in CS and HS, but others are specific for each type: for instance only CSs present GlcA-3-O-sulphation. Other modifications consist in 4-O- and 6-O-sulphation of GalNAc, and 2-O-sulphation of the uronic acid (Zhang, 2010).

CSs are typically involved in structural and regulatory activities. For instance, as components of the ECM, they are important for the integrity of tissues like cartilage (Dijkgraaf *et al*., 1995). In the CNS, CS have a regulatory role: they are for instance the fundamental components of the perineuronal nets (PNNs) in the brain and in the visual cortex. PNNs are specialized structures of the ECM which have a role in the closure of critical periods. During the critical period, sensory experience shapes the organization of the neuronal network, which is plastic at this stage. For example, in the visual cortex of young rats the PNN is not completely formed: this allows plasticity and correct organization of the neuronal connections. However, the cortex of adult rats with a well-developed PNN is less plastic. This is partially due to CSPGs which are present in adult rats, but not in younger animals. It has been shown that digestion of CSPGs with chondroitinase ABC restores the neuronal plasticity in adults (Pizzorusso *et al*., 2002). However, some CS have apparently different functions: a class of CS (CS-E) is instead promoting axonal growth in a cell-specific fashion (Mikami and Kitagawa, 2013).

GDNF binds CS with high affinity (10-fold higher than affinity to HS) and with almost no dissociation. It has therefore been proposed that CS could keep holding neurotrophic factors while presenting it to the receptor, or that growth factors could be enzymatically released from such molecules (Nandini *et al*., 2004). However, CS does not seem to be important for GDNF signalling, as this is not decreased in cells lacking CSPGs, and exogenously added CS had only a slight effect on GDNF activity (Barnett *et al*., 2002).

4.2 Heparan sulphate proteoglycans (HSPGs)

Synthesis of HSPGs starts in the same way as that of CSPGs. The difference arises when the fifth sugar is added to the linker sequence: in the case of HSs this is GlcNAc, and the repeat-